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PRINCIPAL INVESTIGATOR: Wende M. Kozlow, M.D.

CONTRACTING ORGANIZATION: University of Virginia
Charlottesville, Virginia 22904

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Wende M. Kozlow, M.D.

E-Mail: wmk8t@virginia.edu

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**14. ABSTRACT**

Aromatase inhibitors (AIs), effective treatment for breast cancer, block estrogen synthesis. Increased bone resorption and decreased bone mineral density (BMD) are predicted consequences. We hypothesized that bisphosphonates (BPs) may prevent bone loss from AI therapy. Four-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole + ZA or control. Mice treated with letrozole alone had lower BMD compared to control ($p<0.0001$; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control ($p<0.0001$; total body, spine, femur and tibia). MicroCT analysis of the tibia showed no difference in trabecular bone volume (BV/TV) or trabecular number, thickness or spacing in mice treated with letrozole compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV and trabecular number and thickness, and the structural model index indicated that the bone structure was unusually solid. ZA prevented AI-induced bone loss, but microCT and dynamic bone histomorphometry suggest reduced bone remodeling. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

**15. SUBJECT TERMS**
aromatase inhibitors, letrozole, bone metastases, breast cancer, ovariectomy, bisphosphonates, zoledronic acid

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**Effect of a high bone turnover state induced by estrogen deficiency on the development and progression of breast cancer bone metastases.**

## Introduction

Estrogen blockade is the standard medical therapy for treatment of breast cancer and breast cancer metastases. Therapy to suppress estrogen ultimately leads to increased bone resorption and osteoporosis. Cancer treatment-induced bone loss is likely to become the most common skeletal complication of malignancy. Our hypothesis is that breast cancer bone metastases are increased when bone is in a state of high turnover resulting from estrogen deficiency, and that inhibition of increased bone resorption will reduce the development and progression of breast cancer bone metastases. We are using a mouse model to test the effects of a high bone turnover state from estrogen deficiency on breast cancer metastases to bone, and to determine if inhibition of increased bone turnover due to estrogen deficiency will reduce breast cancer metastases to bone.

Breast cancer is the second leading cause of cancer death in women. In 2006, it is predicted that 212,920 women will be diagnosed with invasive breast cancer, and that 40,970 women will die from this disease (http://www.cancer.org/cancerinfo). Breast cancer metastasizes to bone in greater than 80% of patients with advanced disease, causing hypercalcemia, bone pain, fractures and nerve compression (1). Treatment of breast cancer generally involves a combination of surgery, radiation therapy, chemotherapy and endocrine therapy (2).

The growth of many breast cancers is stimulated by estrogen. Therefore, treatment of breast cancer and breast cancer metastases has focused on mechanisms to block the effect of estrogen at the estrogen receptor (selective estrogen receptor modulators) and to prevent estrogen synthesis (aromatase inhibitors) (3). Aromatase is a member of the cytochrome P450 superfamily; it catalyzes the irreversible conversion of androstenedione and testosterone into estrone and estradiol respectively. Thus, AIs block the rate-limiting step in estrogen biosynthesis, reducing estrogen levels by 90-95% (4;5). Recent data suggest that AIs are more efficacious than tamoxifen. The trial comparing letrozole to placebo in early-stage breast cancer in postmenopausal women showed that letrozole, after treatment with tamoxifen for five years, significantly improved disease-free survival (6). A trial using exemestane after two to three years of tamoxifen in postmenopausal women with primary breast cancer showed a significantly improved disease-free survival as compared to five years of tamoxifen (7).

In contrast to the bone protective effects of tamoxifen, emerging data indicate detrimental effects of AIs on bone. Aromatase deficiency, due to inactivating mutations of the aromatase gene, causes severe demineralization of the skeleton despite increased testosterone levels (8;9). Demineralization was reversible with estrogen. In a model of aromatase deficiency, mice had decreased peak BMD with accelerated bone loss. These changes were reversible with estradiol (10). In the trial comparing letrozole to placebo, there was a new diagnosis of osteoporosis in 5.8% of the letrozole group versus 4.5% in the placebo group (6). The ATAC trial evaluated anastrozole, tamoxifen,

or the combination of anastrozole and tamoxifen for adjuvant treatment of postmenopausal women with early breast cancer. At 37 months, fracture incidence in the anastrozole arm was 7.1% compared to 4.4% in the tamoxifen arm (11). BMD and markers of bone turnover were measured in a subset of patients and compared to control patients with breast cancer not receiving hormone therapy. In the anastrozole arm, there was a decrease in BMD and an increase in the markers of bone turnover. In the tamoxifen arm, there was an increase in BMD and a decrease in the markers of bone turnover (12). Furthermore, studies have reported increased osteoporotic fracture risk in breast cancer survivors (13-15). The effect of AIs on BMD in healthy postmenopausal women has not been studied. These findings, however, suggest that a reduction in postmenopausal estrogen levels from AIs will result in decreased BMD.

The increased bone resorption that occurs as a result of treatment with AIs may increase bone metastases in high-risk women. Increased bone resorption releases growth factors from bone, such as transforming growth factor β (TGFβ), which can then induce breast cancer cell production of osteolytic factors, such as parathyroid hormone-related protein (PTHrP) (16). Osteolytic factors promote bone destruction and tumor growth. BPs, potent inhibitors of bone resorption, reduce skeletal morbidity from bone metastases (17). By decreasing bone resorption, they may reduce the release of factors from bone that stimulate tumor growth. BPs also cause apoptosis of breast cancer cells in vitro (18;19), decrease invasion of bone matrix (20) and decrease adhesion in vitro (21;22). The BP zoledronic acid inhibits angiogenesis both in vitro and in vivo (23). It is unknown whether BPs used concomitantly with AIs will suppress bone turnover and prevent bone metastases. The answer to this question, however, will have important therapeutic implications for the skeletal health and quality of life for women with breast cancer.

## Body

**Hypotheses**:

**1) Bone metastases are increased when bone is in a state of high turnover caused by estrogen deficiency.**

**2) Inhibition of increased bone turnover will prevent breast cancer bone metastases.**

**Specific Aim 1:** To determine the effects of estrogen deficiency, induced by ovariectomy (OVX), on the development and progression of human breast cancer metastases to bone in a mouse model **(hypothesis 1)**.

**Specific Aim 2:** To determine the effects of estrogen deficiency, induced by treatment with the AI letrozole, on the development and progression of human breast cancer metastases to bone in a mouse model **(hypothesis 1)**.

**Specific Aim 3:** To determine if inhibition of the increased bone resorption associated with estrogen deficiency, due to OVX or treatment with an AI, will prevent the development and progression of human breast cancer metastases to bone in a mouse model **(hypothesis 2)**.

## Tasks:

**Task 1 (Specific Aim 1):** months 01-04. Female nude mice will be randomized to OVX or sham surgery. Four weeks post surgery, when decreased BMD was seen in female nude mice after OVX in a previous experiment, intra-cardiac inoculation with the human breast cancer cell line MDA-MB-231 (MDA-MB-231) will be performed in all mice (12 mice/group).
**Task 2 (Specific Aim 1):** months 05-06. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 1.

***For Tasks 1 & 2:*** Forty 4-week-old female nude mice were randomized to OVX or sham surgery. At 8 weeks post surgery, 8 mice from each group were euthanized and 12 mice from each group were inoculated with MDA-MB-231 via the intra-cardiac route. The mice were followed with X-rays at baseline and then at 1-week intervals to monitor the development and progression of bone metastases. BMD was measured at baseline and then every 2 weeks for the duration of the experiment. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

- ***OVX.*** Mice were anesthetized with ketamine/xylazine and placed prone. Ovaries were excised. The mice were sutured and hydrated with 3cc of saline. The incision site was treated with an antibiotic cream and the mice were placed on a warm heating pad until they recovered from anesthesia. Control animals received sham surgeries at the same time.
- ***Bone metastases model.*** Tumor cells were trypsinized and resuspended in PBS to a final concentration of $10^6$ cells/100μL immediately prior to injection. The mice were anesthetized with a ketamine/xylazine mixture and positioned ventral side up. The left cardiac ventricle was punctured through a percutaneous approach using a 26-gauge needle (16;24;25;26). For radiography, the mice were anesthetized deeply, placed prone against the detector, and exposed with an x-ray at 35 kVp for 5 seconds using a Faxitron Unit.
- ***Analysis of metastases.*** All radiographs from the mice were evaluated in a blinded fashion. The number and area of osteolytic bone metastases were calculated on radiographs using a computerized image analysis system (16;25;26). Quantification of lesion area and number was performed using image analysis software (Metaview/Metamorph Software). This system detects lesions as small as 0.1mm.
- ***Bone & soft tissue histology & histomorphometry.*** Forelimb and hindlimb long bones, spine, calvaria and soft tissues were harvested, fixed in 10% buffered formalin, decalcified in 10% EDTA and paraffin embedded. Sections for histomorphometric analysis were stained using hemotoxylin and eosin, orange G and phloxine. The following variables were measured in sections of bone to assess bone resorption (16;25;26): total bone area, total tumor area, osteoclast number per mm of tumor-bone interface and histomorphometry (using Metaview/Metamorph). In soft tissue blocks, tumor area will be measured to determine if estrogen suppression alters metastases to non-bone sites.
- ***Insulin-like growth hormone-1 (IGF-1) serum levels.*** Whole blood was collected by retro-orbital puncture. The collected serum was aliquoted into 20 μl

samples and stored at -80°C. IGF-1 was measured using IGF-1 RIA (American Laboratory Products Company, Windham, NH). Calculated assay sensitivity is 0.02 ng/mL; cross reactivity with IGF-2 is <0.05%. Inter-assay CV is 6%; intra-assay CV is 4%. IGF-1 was dissociated from IGF-1 binding proteins by dilution in acidic buffer, and an antibody solution containing excess IGF-2 was added to neutralize samples. IGF-1 was then measured with addition of $^{125}$I tracer.

- ***BMD measurements.*** BMD was measured in anesthetized mice using a Lunar Piximus. Total body, lumbar spine, mid-femur, proximal femur and proximal tibia BMD was done at baseline and then at 2-week intervals.
- ***Body composition measurements.*** Body composition was measured in anesthetized mice using a Lunar Piximus. Percent fat mass and fat mass were measured at baseline and then at 2-week intervals.
- ***Statistics.*** Data was analyzed by ANOVA followed by Tukey-Kramer multiple comparison test for comparing > 2 groups and by the Student's t-Test for comparison of 2 treatment groups.

***Results for tasks 1 & 2:***

- In a previous experiment, decreased BMD was seen in female nude mice four weeks after OVX. However, 8 weeks after surgery there was no difference in BMD in OVX mice compared to sham mice at any site: total body (p=0.6814), spine (p=0.3398), femur (p=0.3914) or tibia (p=0.3093) **(figure 1)**.
- Histomorphometry showed no difference in trabecular bone volume (TBV) in OVX mice compared to sham mice at the femur (p=0.8634) or tibia (p=0.1329) **(figure 2)**.
- There was no difference in serum IGF-1 levels in the OVX mice compared to the sham mice **(figure 3)**.
- In the mice inoculated with MDA-MB-231 via intra-cardiac injection, there was no difference in total body x-ray lesion area (p=0.4728) or tibia plus femur x-ray lesion area (p=0.4412) between the OVX and sham mice **(figure 4)**.
- There was also no difference in survival between the OVX and sham mice (p=0.0874) after intra-cardiac injection with MDA-MB-231 **(figure 5)**.

***Conclusions, potential problems and alternative strategies for tasks 1 & 2.*** As in our previous experiment, we expected to see decreased BMD in OVX mice compared to sham mice. However, we saw no difference in BMD between the 2 treatment groups. In addition, there was no difference in skeletal metastases or survival in the OVX mice compared to the sham mice after intra-cardiac injection with MDA-MB-231. A possible explanation for this is the genetic heterogeneity of our female nude mice, which are a random mix of the Balb/C and ICR Swiss breeds. This heterogeneity may explain why we have seen a different response to OVX in this experiment compared to our prior experiment. Inbred mice are known to have a variable response to OVX. For instance, Bouxsein *et al* used 5 strains of inbred mice to study the skeletal response to OVX (27). Four-month-old female mice underwent OVX or sham surgery and were euthanized 4 weeks later. The 5 strains of mice varied in terms of the site in which loss of BMD was noted, and in whether or not they lost more trabecular versus cortical bone at each site.

Li *et al* used 3 inbred mouse strains to show that genetic background influences the rate of cortical bone loss after OVX (28). Both Bouxsein *et al* and Li *et al* used older mice (16-week-old) for OVX versus sham studies. Skeletal response to OVX may differ based on age of the mice. Therefore, we have elected to repeat this experiment. The first group of 24 mice will be randomized to OVX or sham surgery at age 4 weeks, and a second group of 24 mice will be randomized to OVX or sham surgery at age 16 weeks. This will allow us to study the skeletal response to OVX in young versus old female nude mice. We will follow BMD every 2 weeks. Once there is a difference in BMD between the OVX and sham mice, 12 mice from each group will be randomized to intra-cardiac injection with MDA-MB-231 or control. It should be noted that younger mice respond more favorably to intra-cardiac injection with MDA-MB-231. Young mice will develop more bone metastases (and at a faster rate) compared to older mice, which is why the 4-week-old time point for OVX was originally chosen. This current experiment will allow us to directly compare the number and speed at which bone metastases develop in younger versus older female nude mice after intra-cardiac injection with MDA-MB-231.

**Task 3 (Specific Aim 2):** months 07-10. Female nude mice will be randomized to therapy with the AI letrozole versus control, administered via subcutaneous (sc) injection. After 4 weeks of treatment with letrozole or control, each group of mice will be randomized to intra-cardiac injection with MDA-MB-231 or control (12 mice/group).
**Task 4 (Specific Aim 2):** months 11-12. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 3.
**Task 5 (Specific Aim 3A):** months 13-16. Female nude mice will be randomized to OVX or sham surgery or to treatment with letrozole or control. At time of OVX/sham surgery or initiation of letrozole/control therapy, the mice in each of the 4 treatment groups will be randomized to receive twice weekly sc injections of zoledronic acid (ZA) or control. After 4 weeks, all mice will undergo intra-cardiac injection with vehicle (control for MDA-MB-231)(12 mice/group).
**Task 6 (Specific Aim 3A):** months 17-18. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 5.
**Task 7 (Specific Aim 3B):** months 19-22. Female nude mice will be randomized to OVX or sham surgery or to treatment with letrozole or control. At time of OVX/sham surgery or initiation of letrozole/control, the mice in each of the 4 treatment groups will be randomized to receive twice weekly sc injections of ZA or control. After 4 weeks, all mice will undergo intra-cardiac injection with MDA-MB-231 (12 mice/group).
**Task 8 (Specific Aim 3B):** months 22-23. Analyze bone (x-ray and BMD) and tumor (x-ray, bone histology and histomorphometry) parameters from mice in Task 7.
**Task 9 (Specific Aims 1-3):** months 24-36. Analyze data and prepare manuscripts and reports.

***For tasks 3-9 (Part 1):*** To study the effects of AIs on bone, and whether or not the skeletal effects of AIs on bone could be prevented with concomitant treatment with the BP ZA, 40 4-week-old female nude mice were randomized to treatment with: 1) control, 2) letrozole, 3) ZA or 4) letrozole + ZA (10 mice/group). Mice were euthanized after 14 weeks of treatment.

- ***Treatment of mice with an AI.*** Mice were treated with letrozole 10 mcg/day/sc starting on day zero and continuing through the end of the experiment. Control mice were administered the same volume of vehicle/day/sc.
- ***Treatment of mice with a BP.*** Mice were treated with 5 mcg/kg/sc of ZA twice weekly, starting on the day zero and continuing through the end of the experiment. The dose of ZA was determined by previous experiments in the Guise laboratory. Control mice were injected with the same volume of vehicle sc twice weekly.
- ***Bone & soft tissue histology & histomorphometry.*** Please see above.
- ***IGF-1 serum levels.*** Please see above.
- ***IGF-1 PCR.*** Total RNA was extracted from homogenized mouse liver in Trizol reagent and treated with DNase prior to quantitative PCR (qPCR). cDNA was generated using an oligo dT-primed Qiagen Omniscript RT kit. qPCR was then performed using a Qiagen SYBR green PCR kit and Bio-Rad MyiQ Single Color Real-time PCR Detection System (Bio-Rad, Hercules, CA).
- ***BMD measurements.*** Please see above.
- ***Micro-computed tomography (micro-CT).*** Micro-CT 40 (Scanco Medical, Bassersdorf, Switzerland) was used to assess skeletal changes in the right proximal tibia from each mouse. Variables measured included bone volume (BV), total volume (TV), calculated ratio of BV/TV, trabecular thickness (Tb.Th), trabecular number (Tb.N), trabecular spacing (Tb.Sp), structural model index (SMI), geometrical degree of anisotropy (DA) and calculated connectivity density (ConnD).
- ***Mechanical loading.*** MTS 858 Bionix materials test system (MTS Systems Corp, Eden Prairie, MN) was used to analyze the right tibia and femur from each mouse. Variables measured included peak load and stiffness.
- ***Dynamic histomorphometry.*** Dynamic histomorphometry was used to assess bone formation rate (BFR) & mineral apposition rate (MAR). On days 1 and 7, mice underwent intraperitoneal (IP) injection with calcein 0.02 mg/gm body weight. On day 4, the mice underwent IP injection with tetracycline 0.03 mg/gm body weight. The mice were then euthanized on day 10. Lumbar spines were embedded in methyl-methacrylate. Seven-micrometer-unstained longitudinal sections were cut and analyzed by epifluorescence microscopy. The histomorphometric examination was performed using Metamorph software and a Leica microscope. All trabecular bone measurements were made at ×200 magnification.

- ***Statistics.*** Please see above.

***Results for tasks 3-9 (Part 1):***

- After 13 weeks of treatment, mice treated with letrozole alone had lower BMD compared to control ($p<0.0001$; total body, spine, proximal femur and tibia) **(figures 6 & 7)**. In the mid-femur, however, mice treated with letrozole alone, as compared to control, did not have lower BMD after 13 weeks of treatment ($p=0.0961$), but a difference in BMD was seen after 7 weeks of treatment ($p=0.0435$) **(figure 8)**. Mice treated with ZA alone had higher BMD compared to control ($p<0.0001$; total body, spine, mid-femur, proximal femur and tibia). Mice treated with letrozole + ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the mid-femur ($p<0.0001$), proximal femur ($p<0.0001$) and total body ($p<0.0023$) **(figures 6 & 7)**.
- Bone histomorphometry demonstrated that mice treated with letrozole alone had the same TBV as mice treated with control at the proximal femur, tibia and lumbar spine **(figure 9)**. Mice treated with ZA (+/- letrozole) had increased TBV compared to letrozole alone at the proximal femur and tibia. In the lumbar spine, mice treated with ZA alone had increased TBV compared to both letrozole ($p<0.05$) and letrozole + ZA ($p<0.05$).
- MicroCT analysis of the proximal tibia showed no difference in BV/TV, SMI, or Tb.N, Tb.Th or Tb.Sp in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV, Tb.N and Tb.Th, and the SMI indicated that the bone structure was unusually solid **(figures 10 and 11)**.
- Dynamic bone histomorphometry of the lumbar spine demonstrated decreased BFR and MAR in mice treated with letrozole, ZA or the combination compared to control **(figure 12)**.
- Mechanical testing showed no difference in peak load or stiffness for either the femur or tibia in the letrozole-treated mice compared to the control mice **(figure 13)**.
- Liver IgF-1 Real-time RT PCR demonstrated decreased IGF-1 expression in letrozole treated mice compared to control ($p<0.01$), an effect that was reversed with the addition of ZA **(figure 14)**. There was no difference in serum IGF-1 levels in mice treated with letrozole alone compared to control **(figure 14)**, although there was decreased serum IGF-1 levels in the ZA alone mice compared to control ($p<0.05$).
- To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation **(figure 15)**.

***Conclusions for tasks 3-9 (Part 1).*** Letrozole decreased BMD in female nude mice, an effect prevented by concomitant treatment with ZA. MicroCT and histomorphometry

analyses indicate that the mechanism involves reduced bone remodeling with no direct effect of the treatment on bone formation. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

***For tasks 3-9 (Part 2):*** To study the effects of AIs on bone without the confounding effects on tumor growth, we used the estrogen-receptor negative breast cancer cell line MDA-MB-231. Therefore, the AI will have no direct effects on the tumor, and any observed effect on bone metastases should be due to the expected increase in bone turnover. Twenty 4-week-old female nude mice underwent inoculation with MDA-MB-231 via intra-cardiac injection. One week later, the mice were randomized to treatment with the AI letrozole or control (10 mice per group). Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

- ***Bone metastases model.*** Please see above.
- ***Treatment of mice with an AI.*** Please see above.
- ***Bone & soft tissue histology & histomorphometry.*** Please see above.
- ***BMD measurements.*** Please see above.
- ***Analysis of metastases.*** Please see above.
- ***Statistics.*** Please see above.

***Results for tasks 3-9 (Part 2):***

- After 3 weeks of treatment, mice treated with letrozole accrued less BMD at the proximal femur (p=0.0161), but achieved the same BMD as control mice at the total body, spine and tibia **(figure 16)**.
- After 4 weeks of treatment, x-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 **(figure 17)**.

***Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 2).***
There was no difference between the letrozole and control groups in terms of changes in BMD or in the development and progression of breast cancer bone metastases after inoculation with MDA-MB-231. The mice either died or were euthanized within 4 weeks of intra-cardiac injection of MDA-MB-231. It may have been too early to see any significant change in bone turnover and, in turn, on the development and progression of breast cancer bone metastases. Therefore, the decision was made to start the letrozole (or control) and then after 4 weeks of treatment, when changes in BMD were seen in an earlier experiment using letrozole and control, inoculate the mice with MDA-MB-231 via intra-cardiac injection.

***For tasks 3-9 (Part 3):*** Twenty-six 4-week-old female nude mice were randomized to treatment with letrozole or control. After 4 weeks of treatment, all mice underwent inoculation with MDA-MB-231 via intra-cardiac injection. Mice were euthanized when they developed significant bone metastases, lost more than 10% of their baseline body weight, or if they showed any signs of distress or impaired mobility.

- ***Bone metastases model.*** Please see above.
- ***Treatment of mice with an AI.*** Please see above.
- ***Bone & soft tissue histology & histomorphometry.*** Please see above.
- ***BMD measurements.*** Please see above.
- ***Body composition measurements.*** Please see above.
- ***Analysis of metastases.*** Please see above.
- ***Statistics.*** Please see above.

***Results for tasks 3-9 (Part 3):***

- After 8 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site **(figure 18)**.
- X-ray analysis demonstrated that there was no difference in total body lesion area between the letrozole-treated mice, as compared to the control mice, after intra-cardiac injection with MDA-MB-231 **(figure 19)**.

***Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 3).*** There was no difference between the letrozole and control groups in terms of changes in BMD or in the development and progression of breast cancer bone metastases after inoculation with MDA-MB-231. We questioned whether or not there was a problem with the letrozole. Novartis kindly supplied us with a new supply of letrozole. In addition, we wondered if the heterogeneity of our female nude mice was contributing to the lack of change in BMD after letrozole treatment. Therefore, we decided to do an experiment to reevaluate the skeletal changes seen in female nude mice after treatment with letrozole versus control.

***For tasks 3-9 (Part 4):*** To study the effects of AIs on bone, 60 4-week-old female nude mice were randomized to treatment with either letrozole or control. After 4 weeks of treatment, 10 mice from each group were euthanized. After 23 weeks of treatment, an additional 10 mice from each group were euthanized. The experiment remains open, with 10 mice in each group. The mice will continue to get BMD measured every 2 weeks until a distinct difference in BMD is noted in at least 2 sites– or until 40 weeks of treatment has been completed. Estrogen deficiency from AI therapy is expected to decrease uterine weight. Therefore, uterine weights were measured after the mice were euthanized to ensure that the letrozole was causing an expected and measurable effect.

- ***Treatment of mice with an AI.*** Please see above.

- ***Bone & soft tissue histology & histomorphometry.*** Please see above.
- ***BMD measurements.*** Please see above.
- ***Body composition measurements.*** Please see above.
- ***Micro-computed tomography (micro-CT).*** Please see above.
- ***Dynamic histomorphometry.*** Please see above.
- ***Statistics.*** Please see above.

***Results for tasks 3-9 (Part 4):***

- After 4 weeks of treatment, there was no difference in BMD between the letrozole and control mice at any site **(figure 20)**.
- After 4 weeks of treatment, there was no difference in TBV in the femur (p=0.2268) or tibia (p=0.9691) of letrozole-treated mice compared to the control mice **(figure 21)**.
- After 4 weeks of treatment, there was no difference in uterine weight or uterine weight/body weight between the letrozole-treated mice compared to the control mice **(figure 22)**.
- MicroCT analysis of the femur and tibia after 4 weeks of treatment did not show a significant difference in trabecular bone volume (BV/TV%), although a trend toward increased BV/TV% in the femurs of letrozole-treated mice was observed (p=0.0659) **(figure 23)**. However, 4 weeks of treatment with letrozole induced marked increases in skeletal microarchitecture. Significant increases in ConnD (p=0.0012) and Tb.N (p=0.0538), Tb.Th (p=0.0280) and Tb.Sp (p=0.0348) were observed in the femurs of letrozole-treated mice, but not in the tibias **(figures 24 and 25)**.
- After 21 weeks of treatment, letrozole-treated mice had increased BMD at the lumbar spine (p=0.0002) and mid-femur (p=0.0030) compared to control mice **(figure 26)**. There was no significant difference in BMD between letrozole and control-treated mice at the total body, proximal femur or tibia.
- After 21 weeks of treatment, letrozole-treated mice had an increased percent change in % fat mass compared to the control mice (p=0.0362), however, there was no significant difference in body weight between the 2 treatment groups (p=0.4522) **(figure 27)**.
- After 21 weeks of treatment, letrozole-treated mice had significantly lower uterine weight (p=0.0148) and uterine weight/body weight (p=0.0107) compared to the control mice **(figure 28)**.

***Conclusions, potential problems and alternative strategies for tasks 3-9 (Part 4).*** Unlike the results of the initial letrozole experiment (tasks 3-9; part 1), differences in BMD were not seen in letrozole-treated mice compared to control mice in the total body,

proximal femur or tibia. However, differences between the treatment groups were seen in the lumbar spine by week 13 and in the mid-femur by week 19. However, in continued contrast to the initial letrozole experiment, the mice randomized to letrozole in this current experiment had higher BMD in the lumbar spine and mid-femur compared to the control mice. In addition, micro-CT data indicated that there was increased trabecular architecture in the femurs, but not in the tibias, of the letrozole-treated mice. Bone histomorphometry and micro-CT data are pending for the 21-week time point of this experiment. There was an increased percent change in % fat mass in the letrozole-treated mice compared to the control mice. In addition, the mice randomized to letrozole initially weighed less than the mice randomized to control (p=0.0006) but, by week 11, there was not a significant difference in body weight between the 2 groups (p=0.0855) **(figure 29)**. The rapid weight gain may have contributed to the increased BMD seen in the lumbar spines and mid-femurs of the letrozole-treated mice.

Letrozole likely has site-specific skeletal effects in the female nude mouse. The genetic heterogeneity of the female nude mouse may be contributing to the conflicting results from these experiments. In addition, the T cell defect in the nude mouse may be complicating the skeletal response to letrozole. Therefore, we plan to use letrozole in an immunocompetent mouse strain in order to clarify the skeletal response to letrozole in mice. OVX produces a variable skeletal response in different inbred mouse strains (27,28), and letrozole may have the same effect.

As noted above, there was an increased percent change in % fat mass in the letrozole-treated mice compared to the control mice. We are in the process of determining if the bones of the letrozole-treated mice have a greater number of adipocytes compared to the bones of control-treated mice. We are also in the process of doing CFU assays to determine the number of adipocytes, osteocytes, osteoblasts and fibroblasts in the bone marrow of letrozole-treated mice compared to control mice.

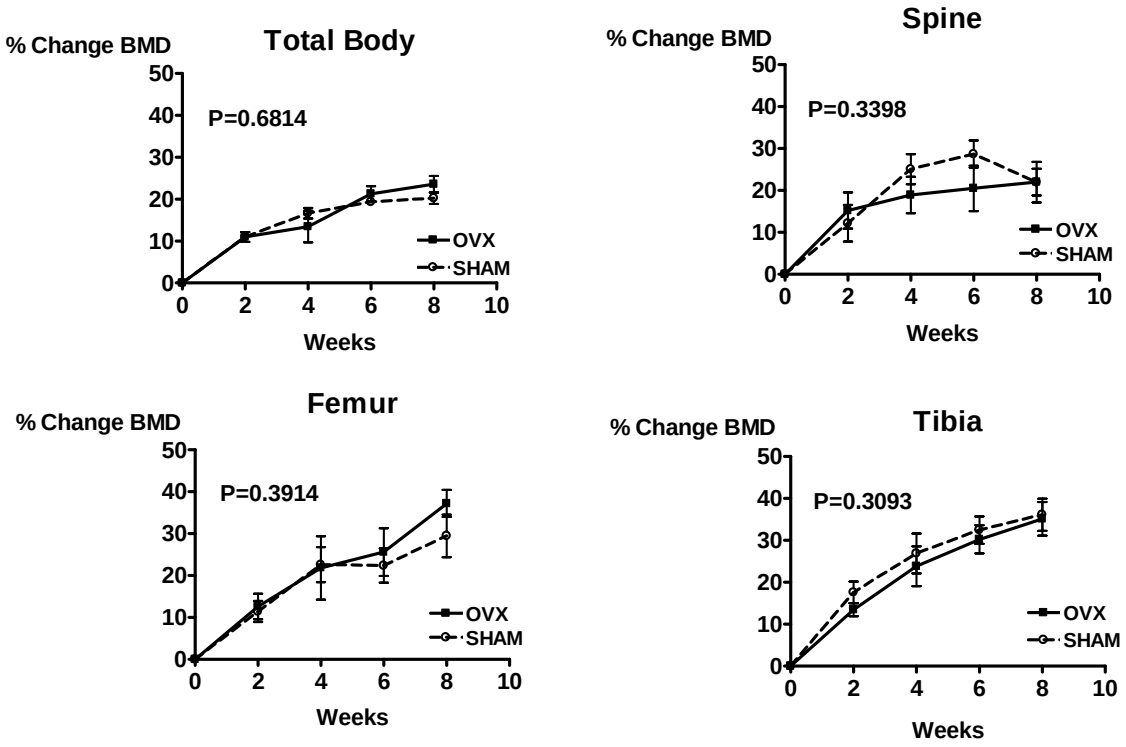



**Figure 1. BMD in female nude mice after OVX versus Sham surgery. There was no difference in BMD between the OVX and Sham mice at any site: A) total body, B) spine, C) femur and D) tibia. P values determined by two-way ANOVA.**

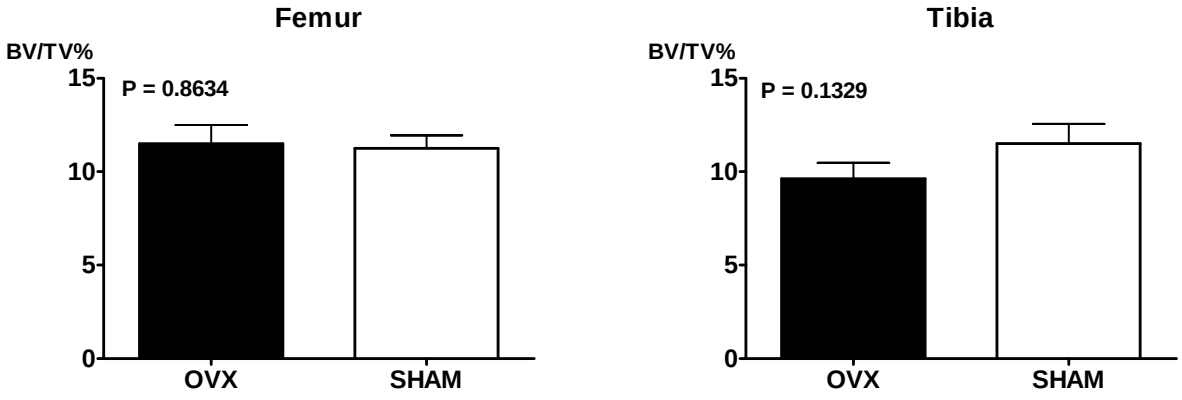



**Figure 2. Trabecular bone volume (BV/TV%) in female nude mice after OVX or Sham surgery. There was no significant difference in BV/TV% between the 2 treatment groups in the proximal femur or proximal tibia. P values determined by two-way ANOVA.**

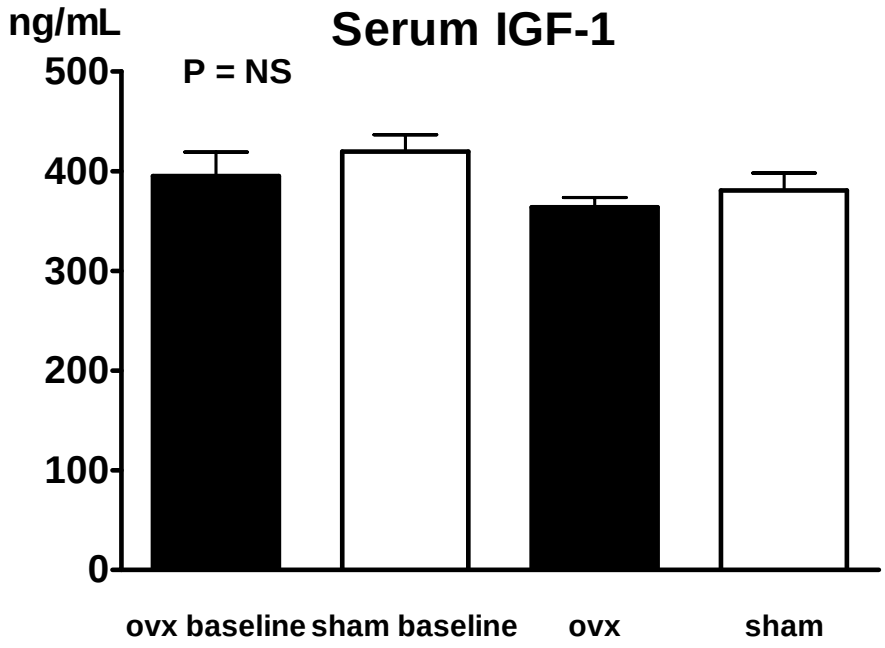



**Figure 3. Serum IGF-1 levels in female nude mice pre and post OVX or Sham surgery. There was no difference in IGF-1 levels in OVX versus sham mice. P values determined by two-way ANOVA.**

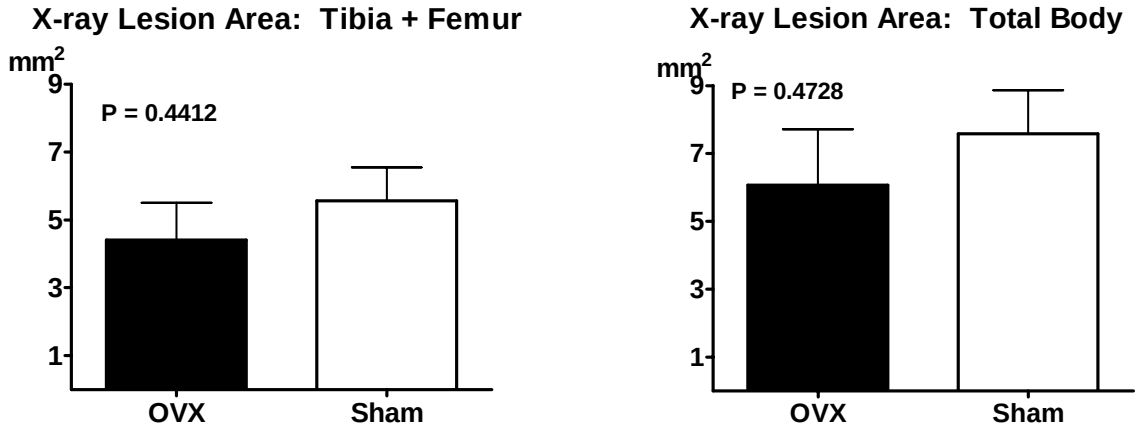



**Figure 4. X-ray lesion area of bone metastases. Female nude mice were inoculated with MDA-MB-231 via intra-cardiac injection 8 weeks after OVX or sham surgery. Lesion area of bone metastases did not differ in the tiba+femur or total body between the OVX or sham treatment groups. P values determined by two-way ANOVA.**

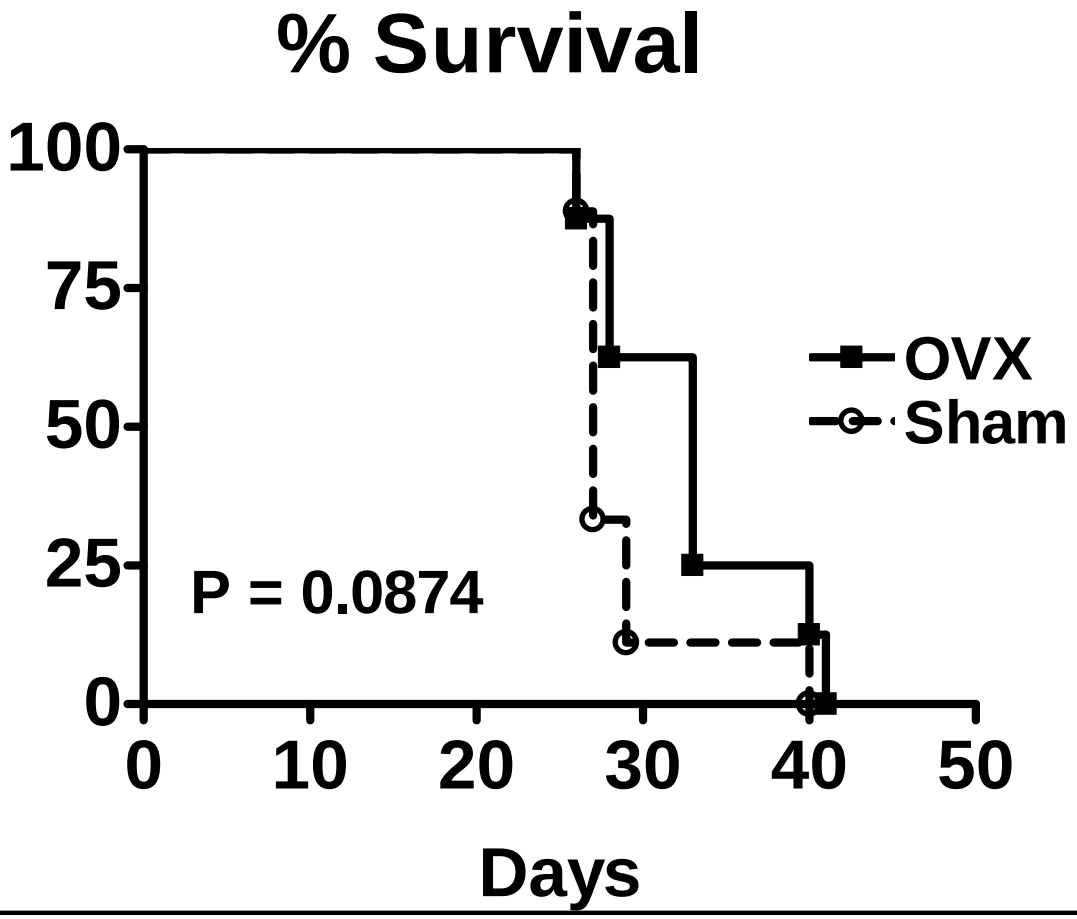



**Figure 5. Survival curve for female nude mice inoculated with MDA-MB-231 via intra-cardiac injection 8 weeks after OVX or sham surgery. There was no difference in survival between the OVX or sham treatment groups.**

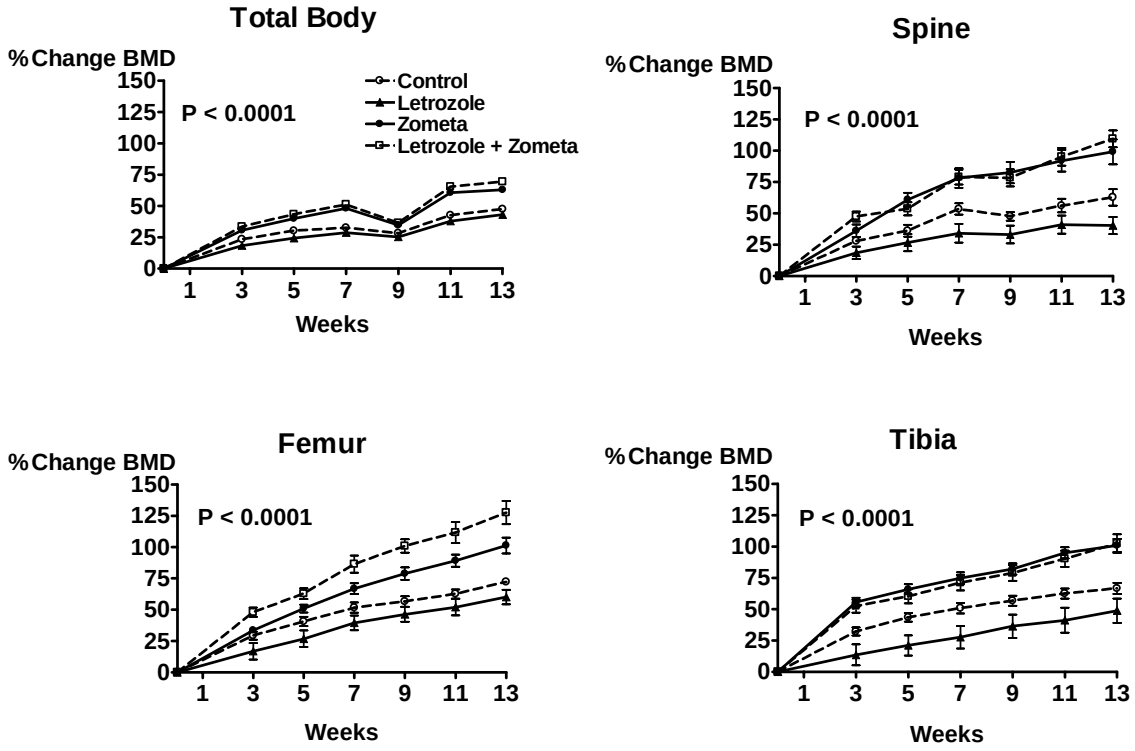



**Figure 6. BMD in female nude mice after 13 weeks of treatment with control, letrozole, zometa (zoledronic acid) or letrozole + zometa: total body, spine, femur and tibia. P values calculated using two-way ANOVA.**

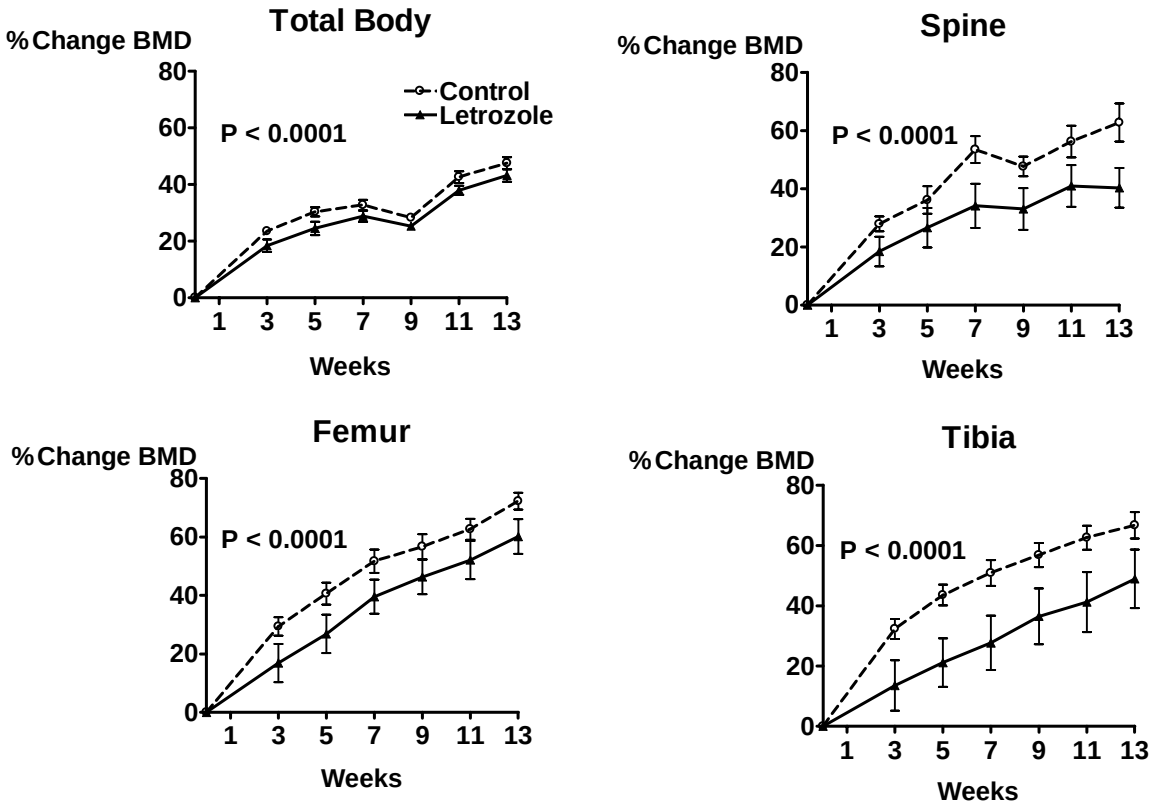



**Figure 7. BMD in female nude mice after 13 weeks of treatment with control or letrozole: total body, spine, femur and tibia. P values calculated using two-way ANOVA.**

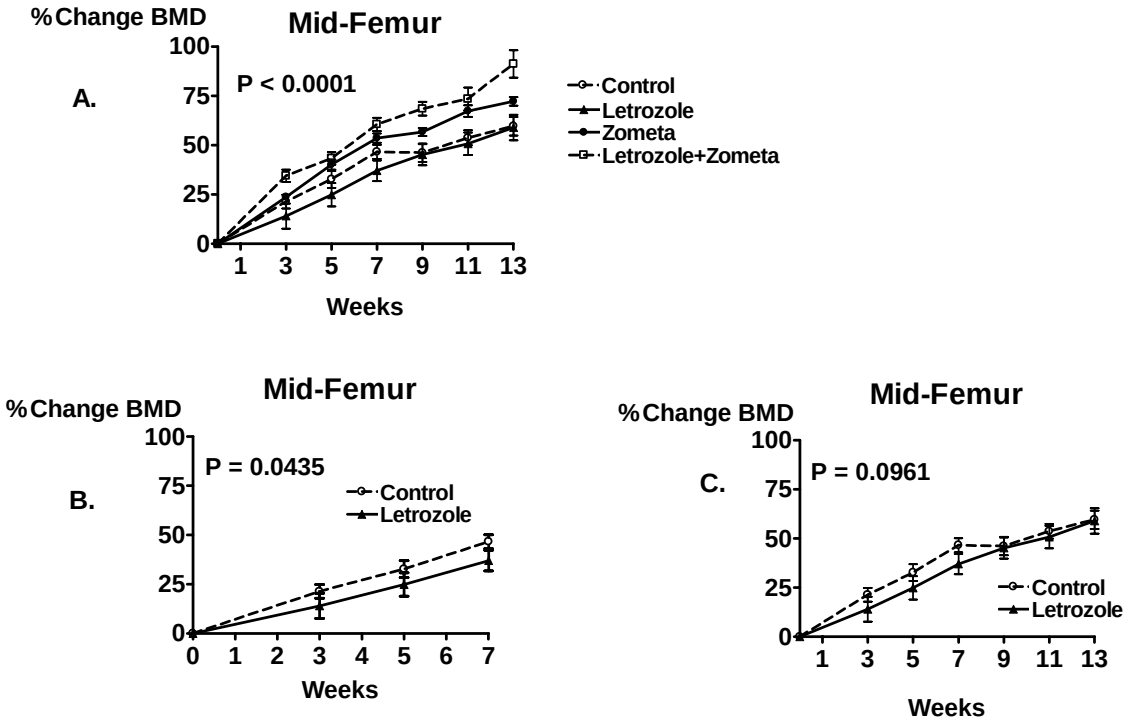



**Figure 8. Mid-femur BMD in female nude mice after A) 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa, B) 7 weeks of treatment with control or letrozole, and C) 13 weeks of treatment with control or letrozole. P values calculated using two-way ANOVA.**

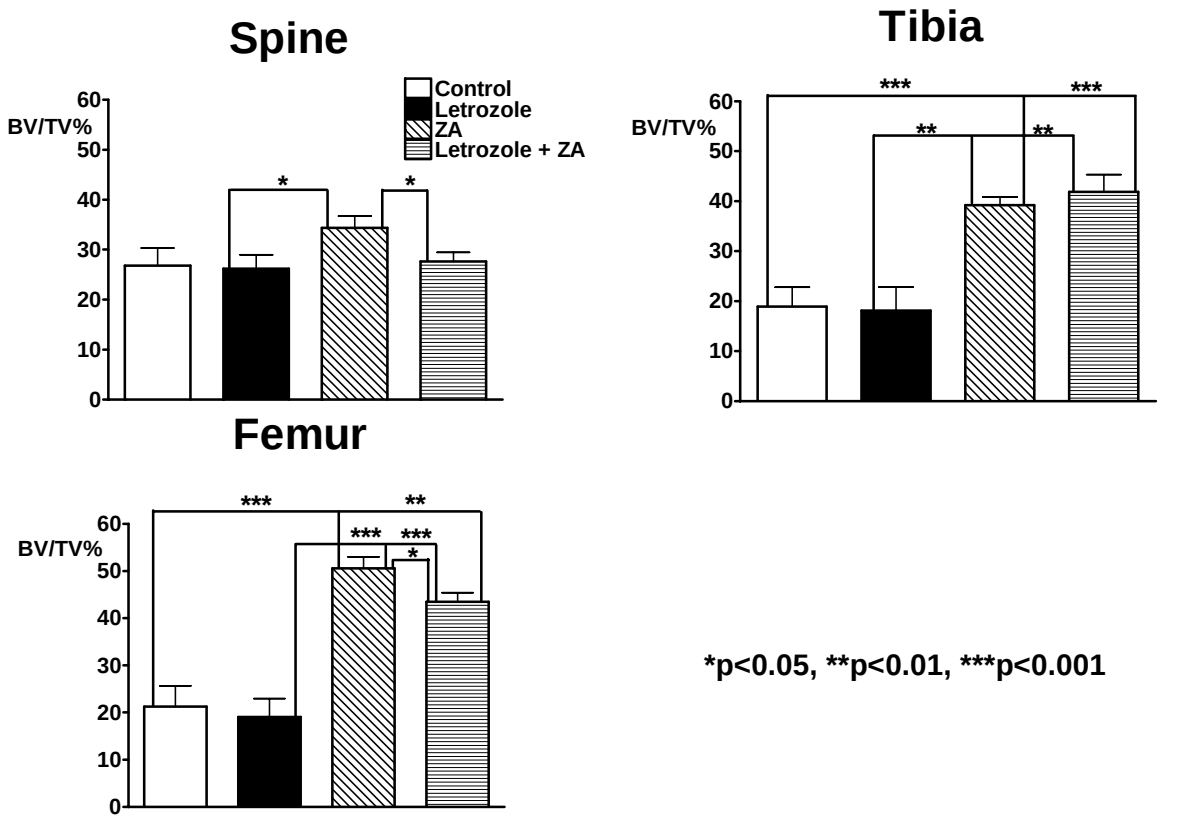



**Figure 9. Trabecular Bone Volume (BV/TV%) in female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa: femur, tibia and spine. There was no difference in BV/TV% between the control versus letrozole group at any site. P values calculated using Student's t-Test.**

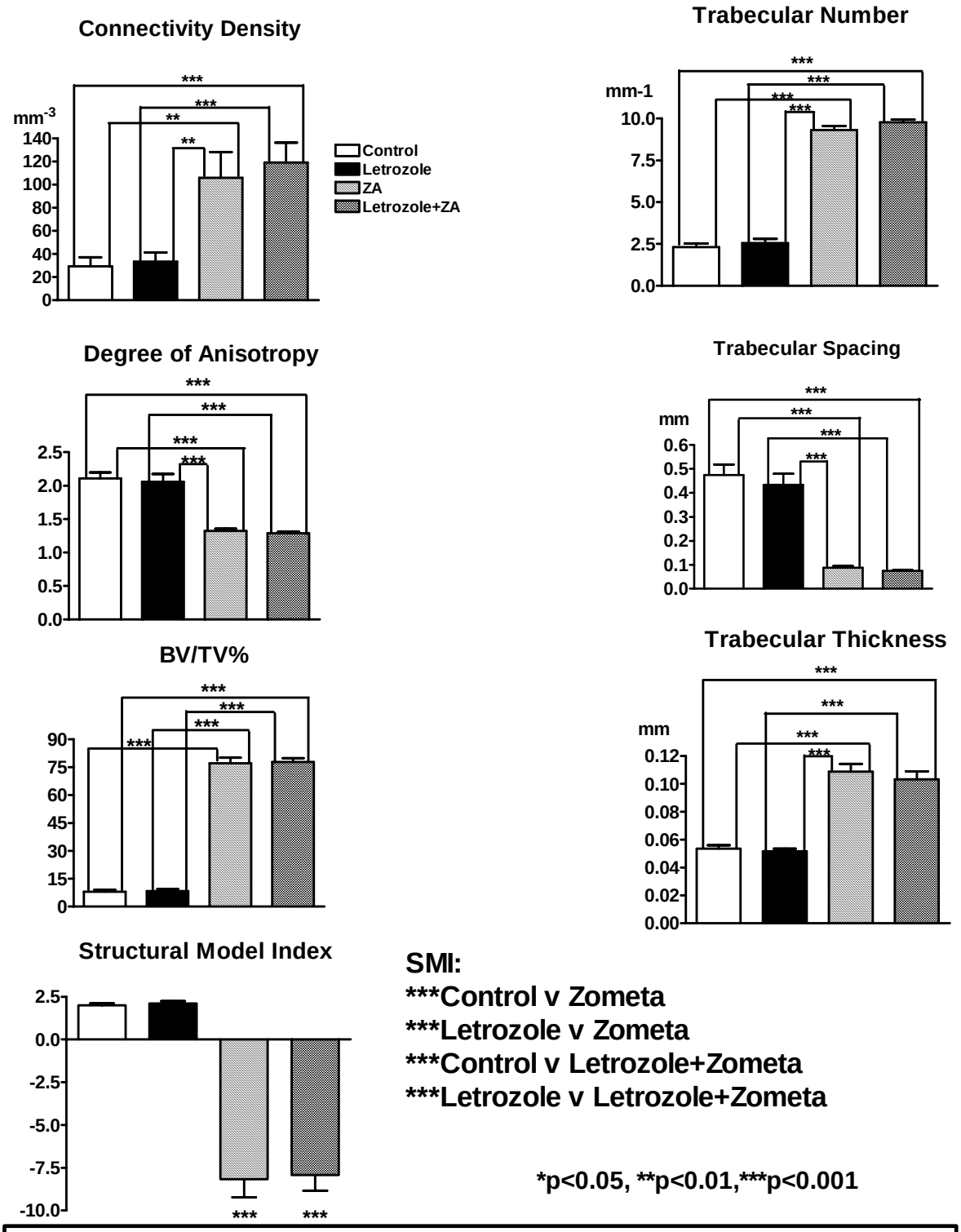



**Figure 10. Micro-CT data from right tibias of female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calcuated using Student's t-Test.**

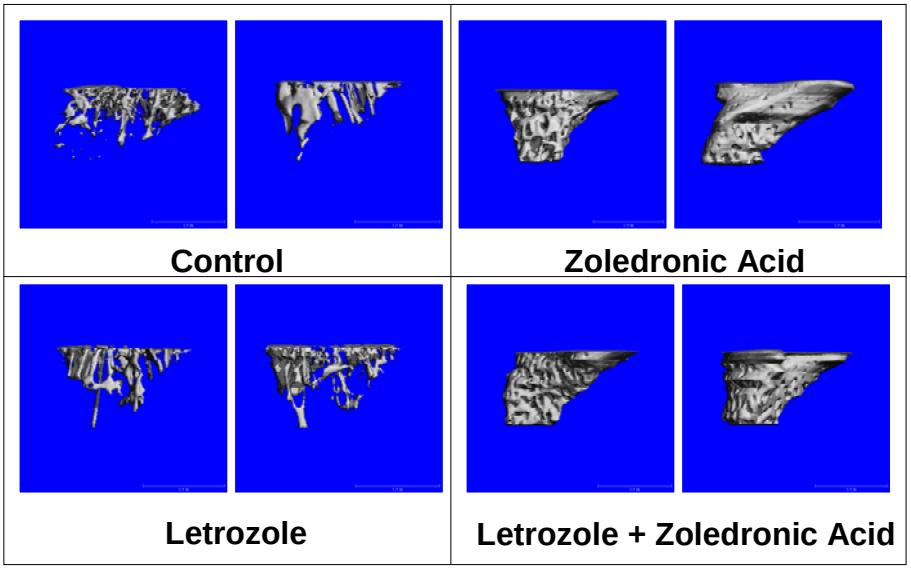



**Figure 11. Micro-CT images of tibias from female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa.**

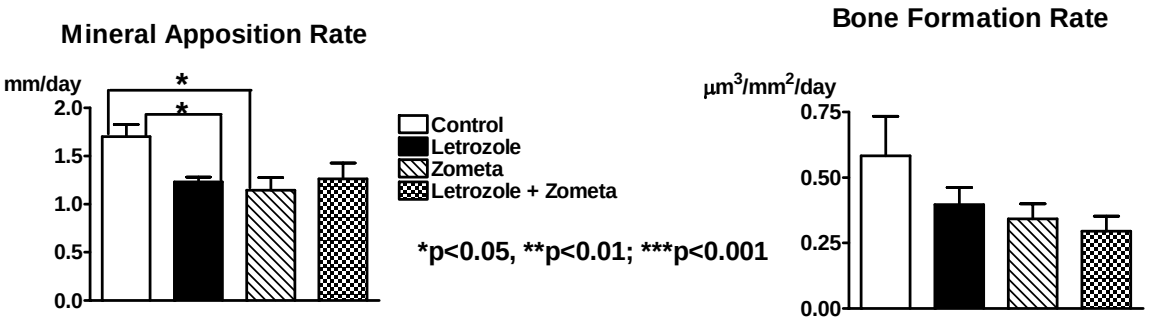



**Figure 12. Mineral Apposition Rate and Bone Formation Rate in female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-Test.**

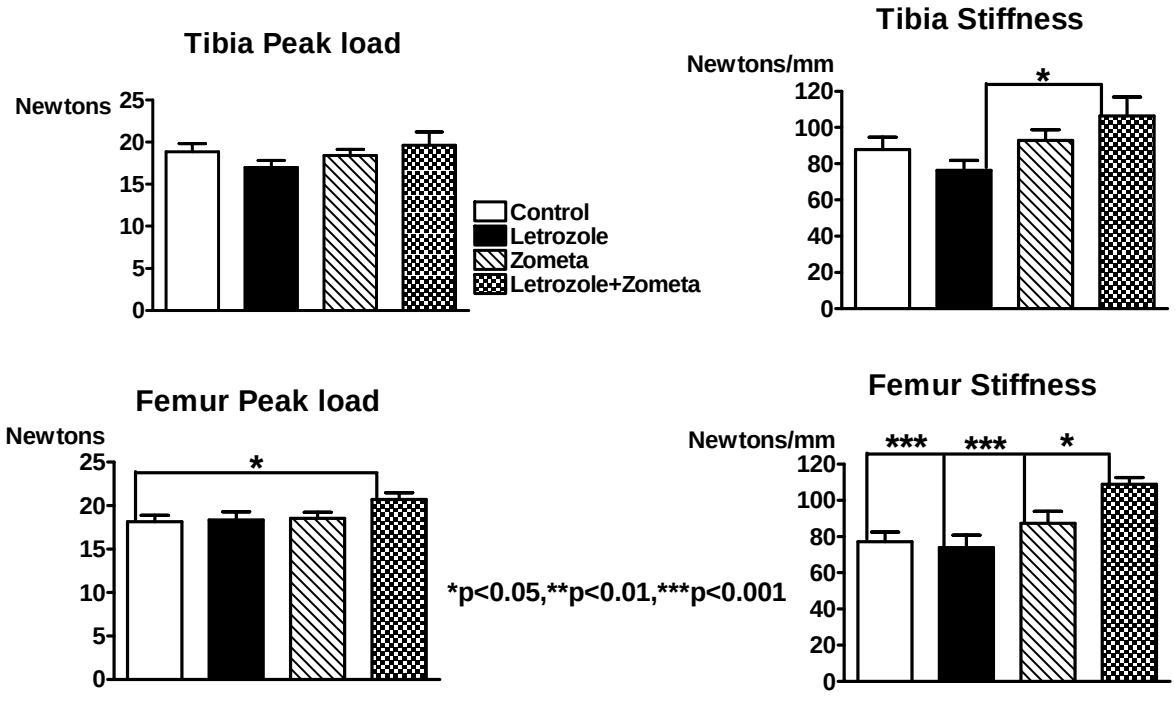
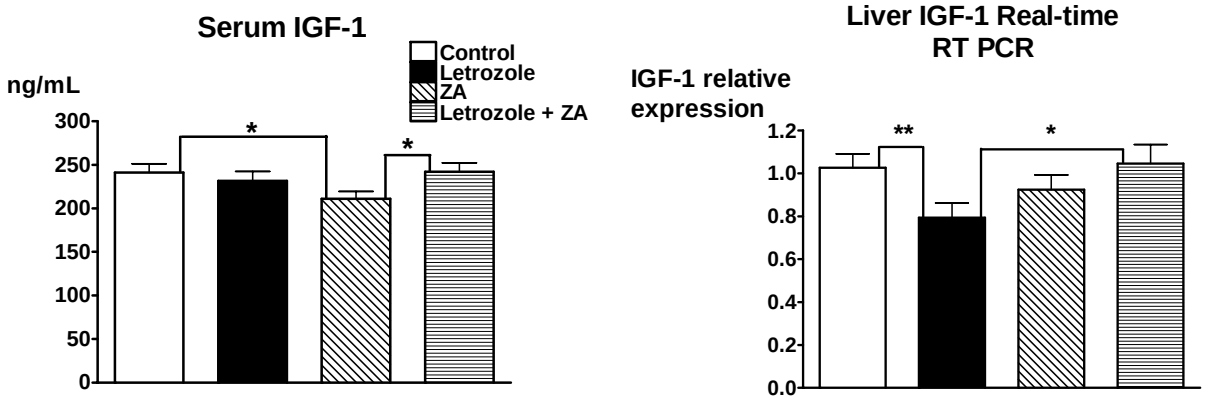



**Figure 13. Mechanical loading data from the right tibias and femurs of female nude mice after 13 weeks of treatment with either control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-Test.**




***p<0.05, **p<0.01, ***p<0.001**

**Figure 14. IGF-1 Real-time RT PCR and serum IGF-1 levels from female nude mice after 13 weeks of treatment with control, letrozole, zometa or letrozole + zometa. P values calculated using Student's t-Test.**

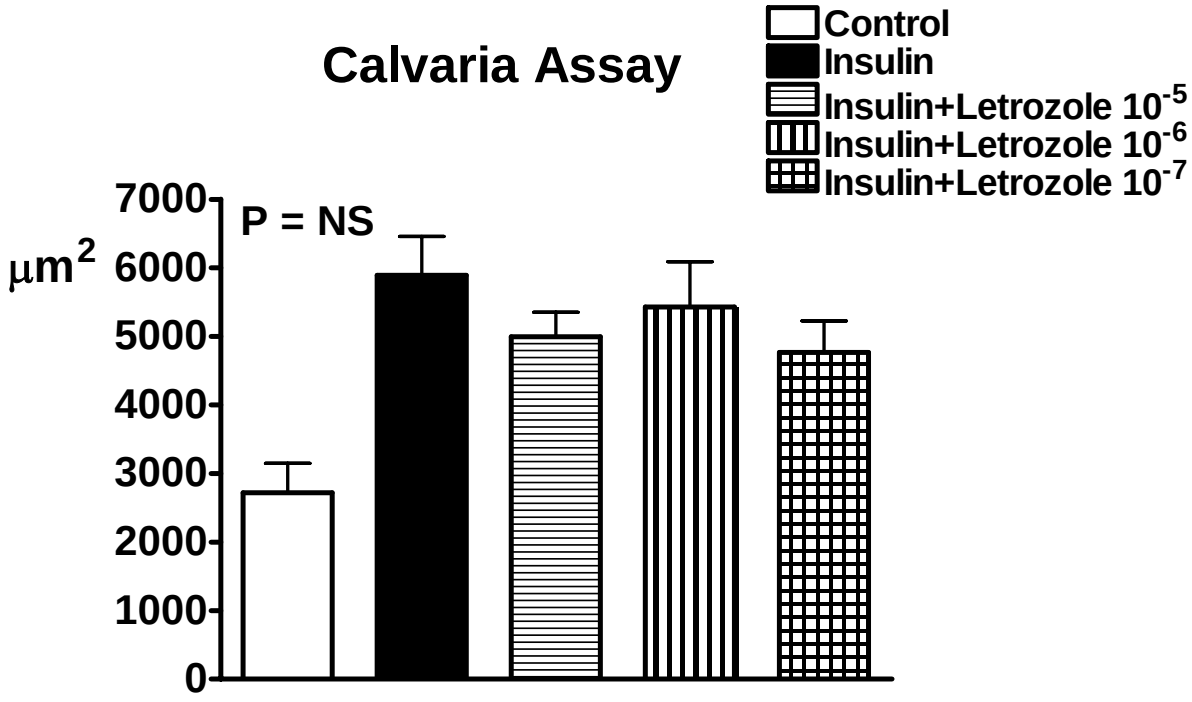



**Figure 15. To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation. P values calculated using Student's t-Test.**

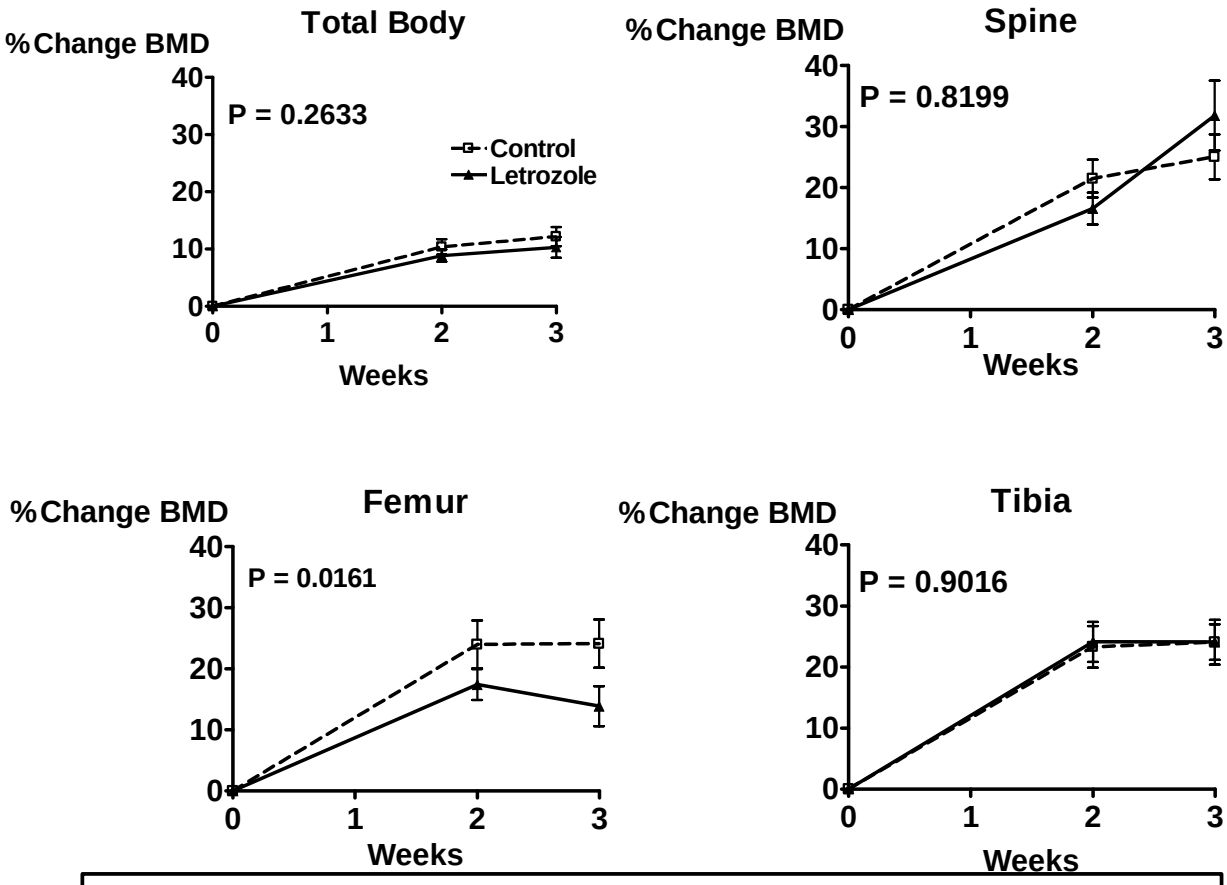



**Figure 16. BMD in female nude mice after 3 weeks of treatment with letrozole or control. P values calculated using two-way ANOVA.**

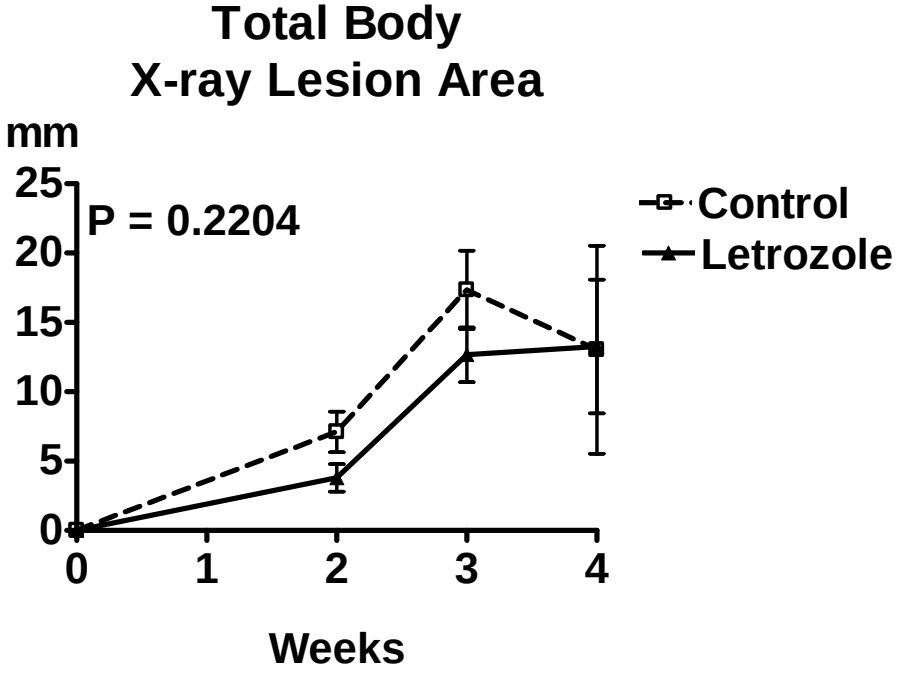



**Figure 17. Total body x-ray lesion area in female nude mice after intra-cardiac injection with MDA-MB-231 and then 4 weeks of treatment with letrozole or control. There was no difference in lesion area of bone metastases between the 2 treatment groups. P values calculated using two-way ANOVA.**

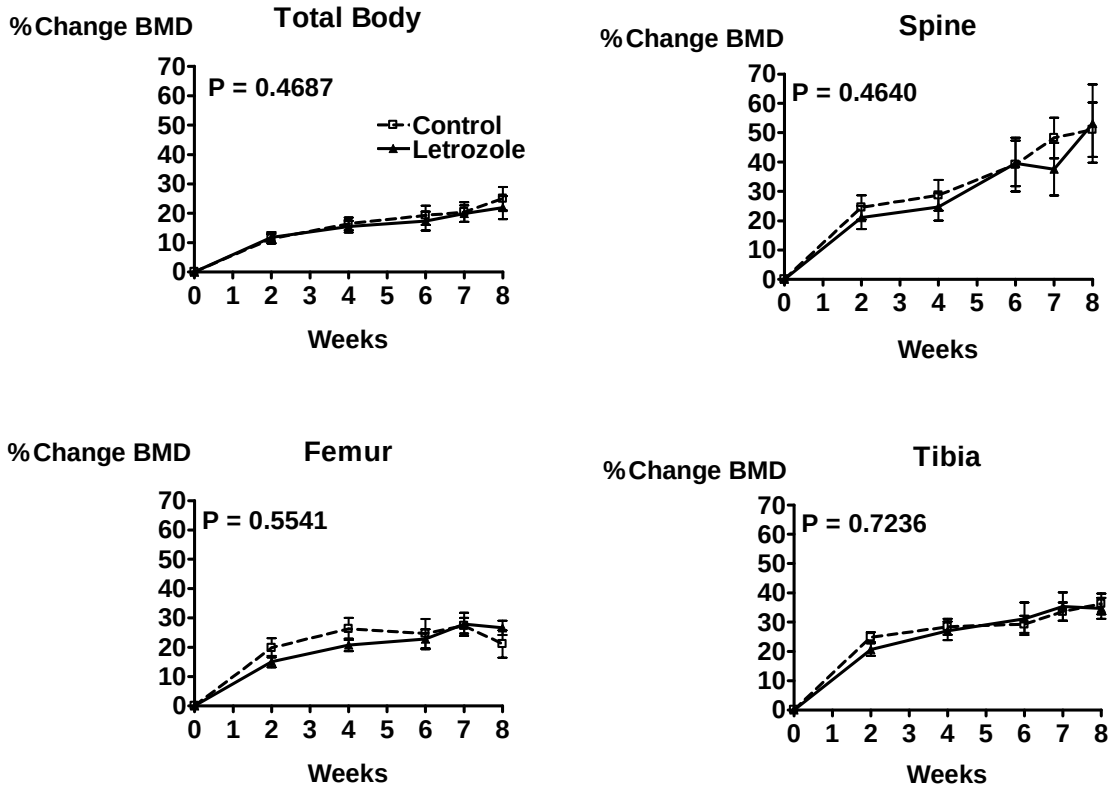



**Figure 18. BMD in female nude mice after 8 weeks of treatment with control or letrozole. Mice treated with letrozole achieved the same BMD as mice treated with control at all sites: total body, spine, femur and tibia. P values calculated using two-way ANOVA.**

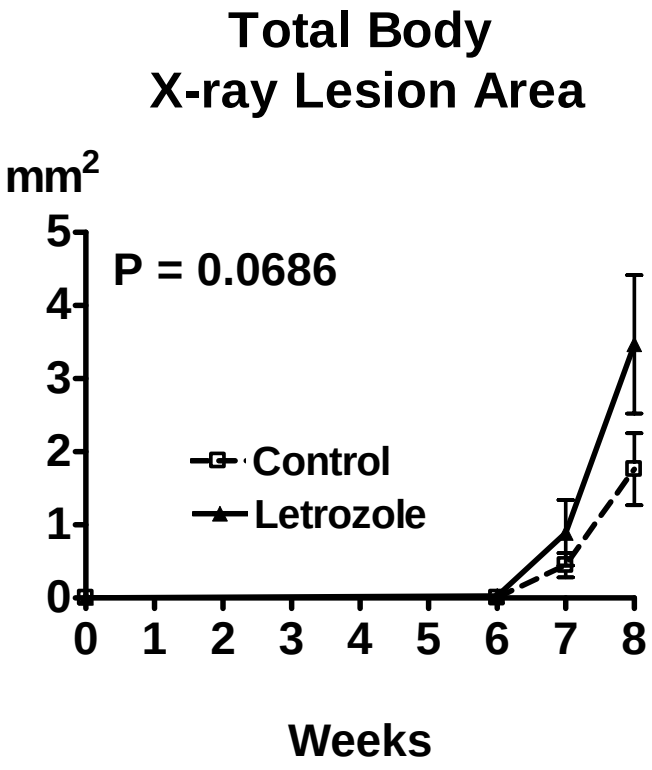



**Figure 19. Total body x-ray lesion area in female nude mice treated for 5 weeks with either letrozole or control, and then inoculated with MDA-MB-231 via intracardiac injection. There was no difference in the lesion area of bone metastases between the 2 treatment groups. P values calculated using two-way ANOVA.**

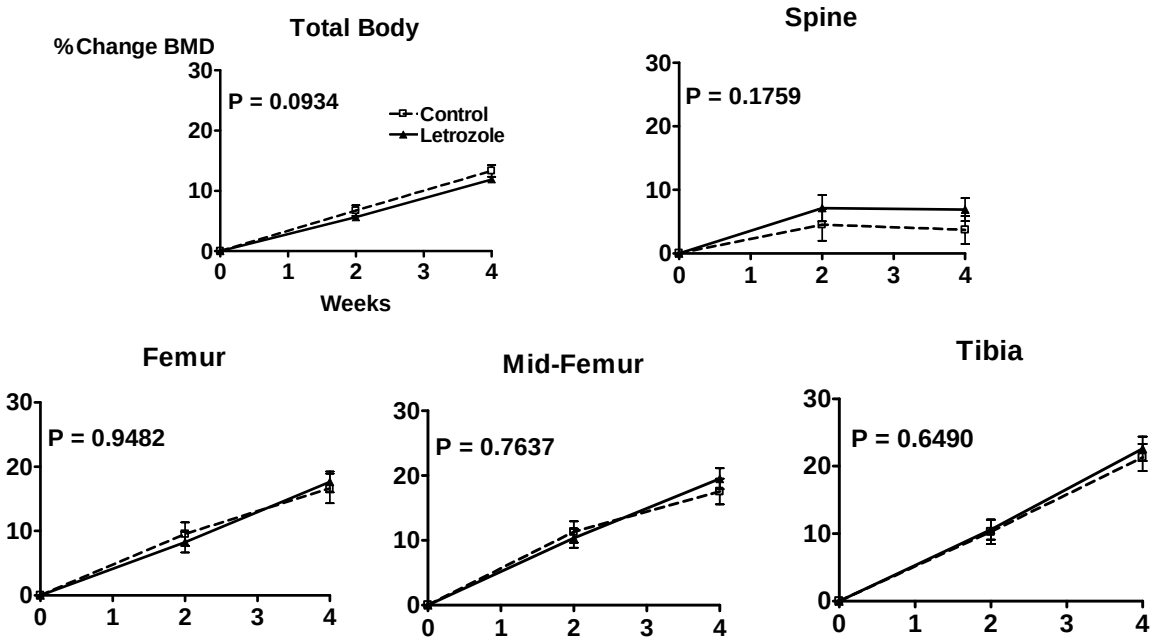



**Figure 20. BMD in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in BMD at any site between the 2 treatment groups. P values calculated using two-way ANOVA.**

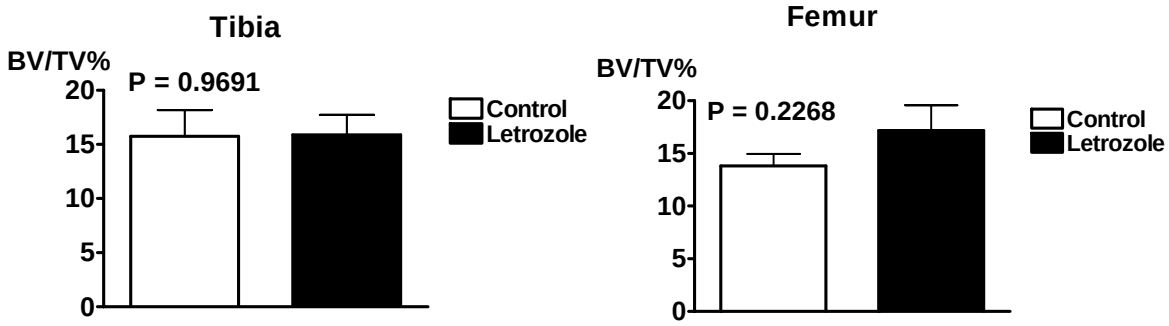



**Figure 21. Trabecular bone volume (BV/TV%) in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in BV/TV% in the tibia or femur between the 2 treatment groups. P values calculated using Student's t-Test.**

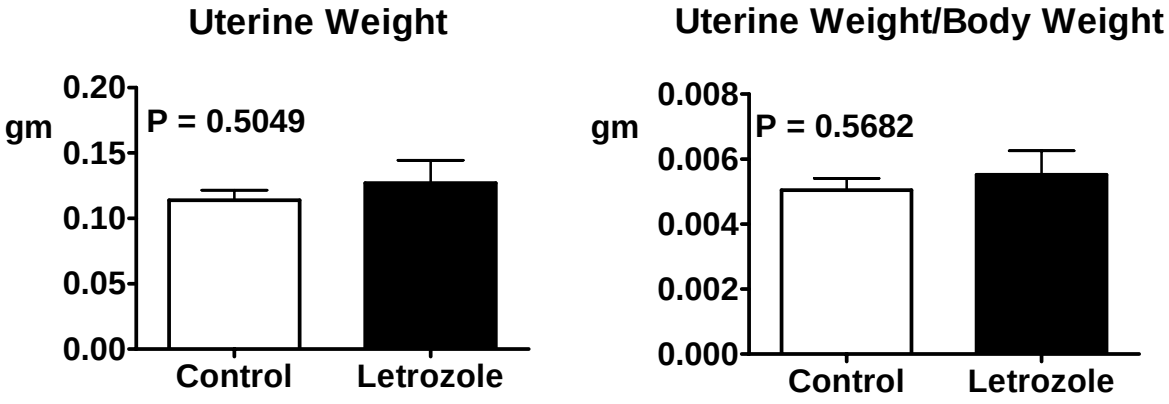



**Figure 22. Uterine weight and uterine weight/body weight in female nude mice after 4 weeks of treatment with letrozole or control. There was no difference in either uterine weight or uterine weight/body weight between treatment groups. P values calculated using Student's t-Test.**

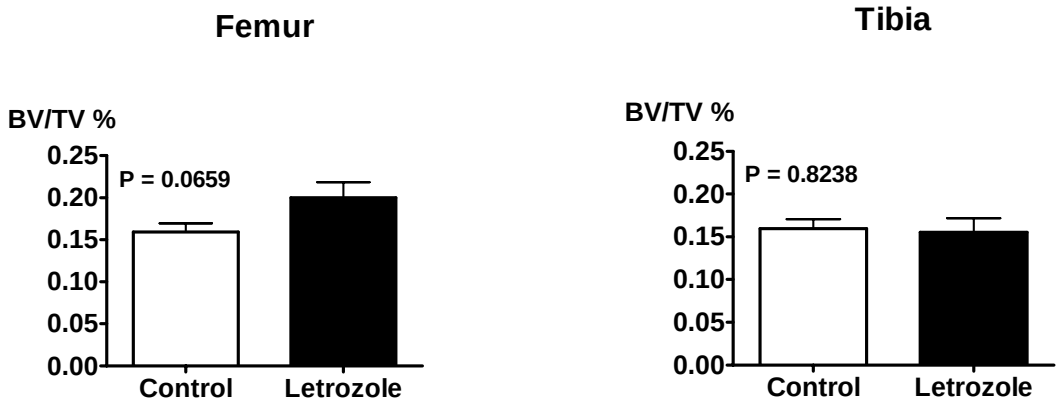



**Figure 23. MicroCT analysis of trabecular bone volume (BV/TV%) of right femur and tibia from female nude mice after 4 weeks of treatment with letrozole or control. There was not a significant difference in BV/TV% in the femur or tibia, although a trend toward increased BV/TV% in the femurs of letrozole-treated mice was observed (p=0.0659). P values calculated using Student's t-Test.**

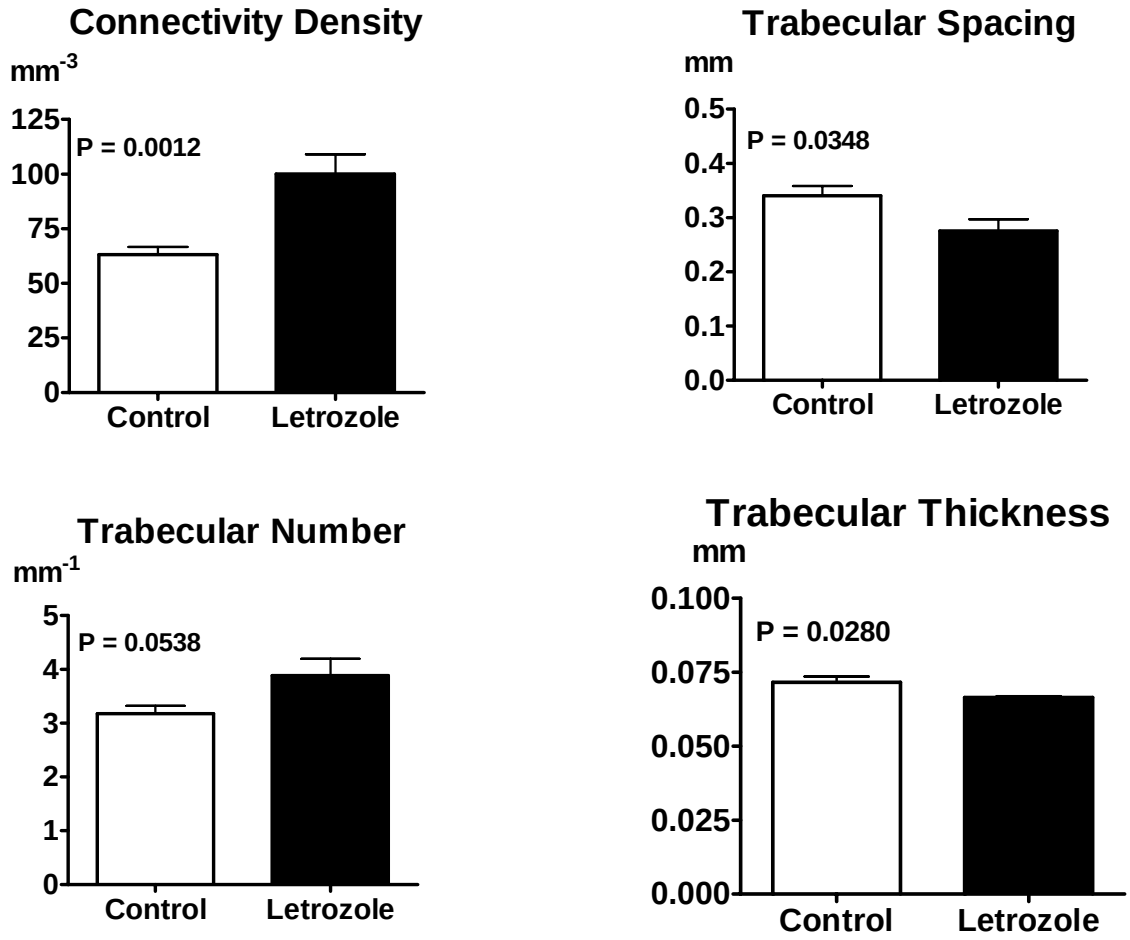
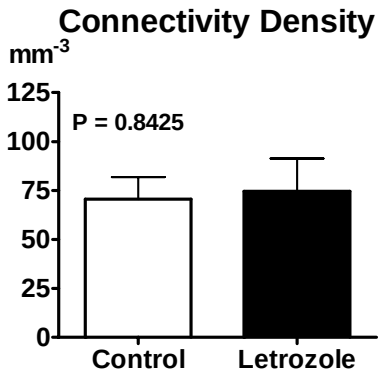
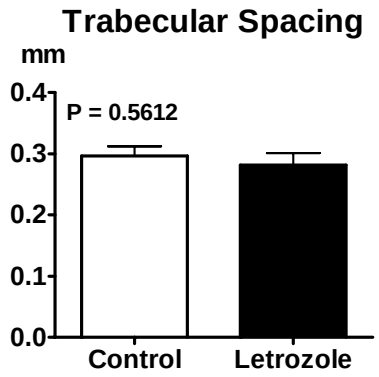
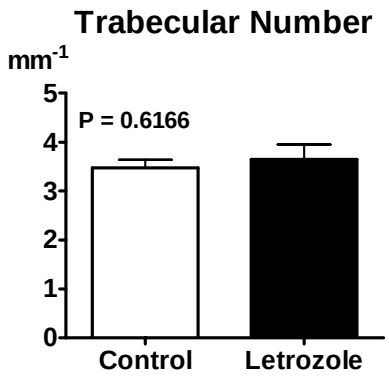



**Figure 24. MicroCT analysis of the right femur from female nude mice after 4 weeks of treatment with letrozole or control. P values calculated using Student's t-Test.**



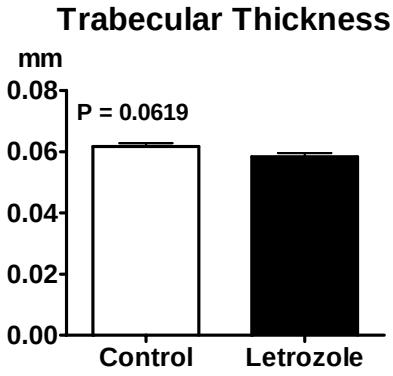




**Figure 25. MicroCT analysis of the right tibia from female nude mice after 4 weeks of treatment with letrozole or control. P values calculated using Student's t-Test.**

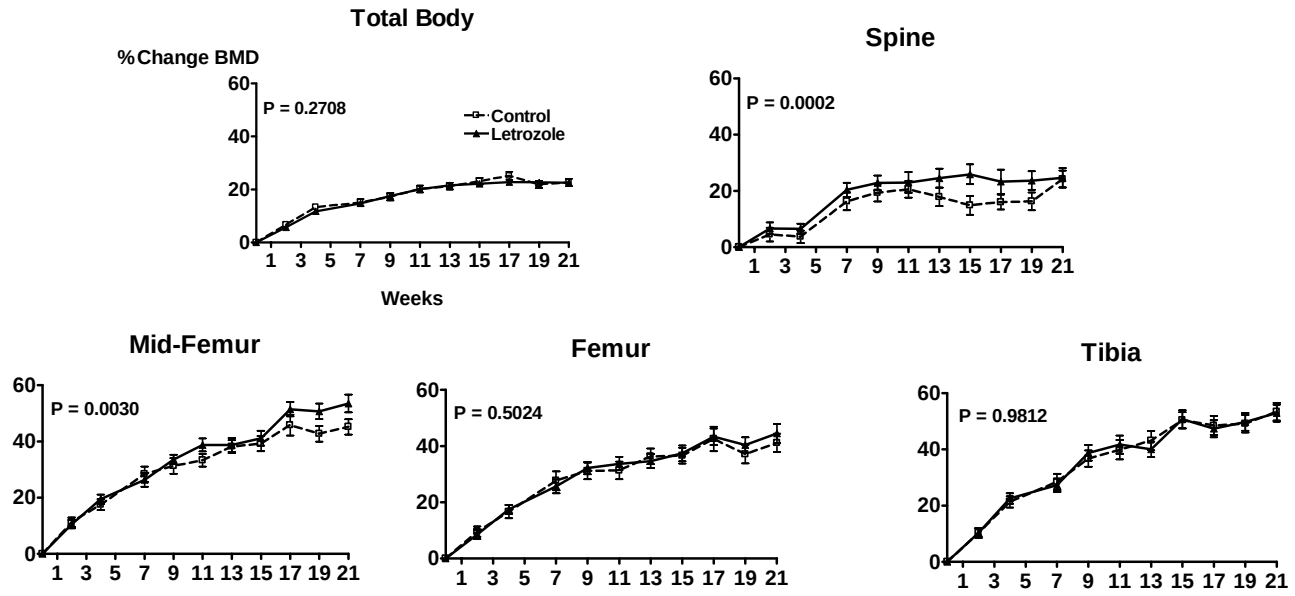



**Figure 26. BMD analysis of female nude mice after 21 weeks of treatment with letrozole or control. After 21 weeks, the letrozole-treated mice accrued more BMD than the control mice at the spine and mid-femur. P values calculated using two-way ANOVA.**

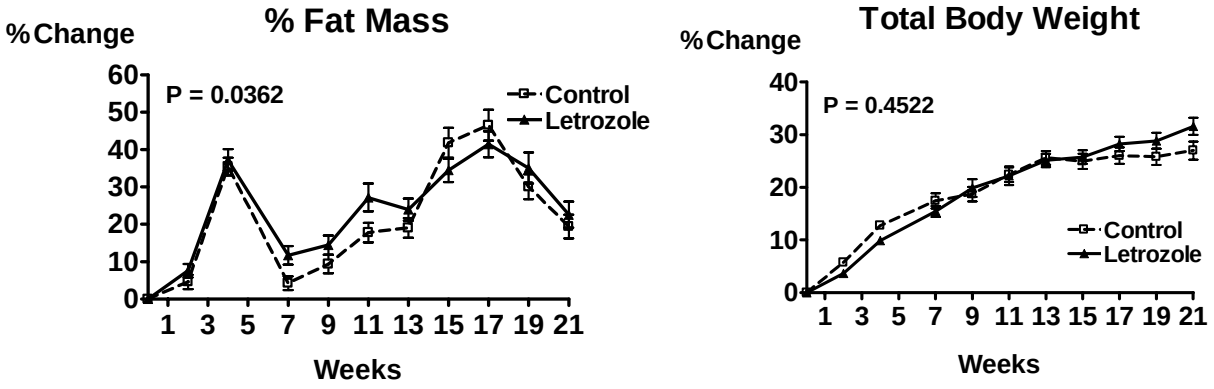



**Figure 27. Percent change of %body fat mass in female nude mice after 21 weeks of treatment with letrozole or control. The letrozole-treated mice have a greater %change of %fat mass than the control mice, although the body weights betweeen the 2 treatment groups did not significantly differ. P values calculated using two-way ANOVA.**

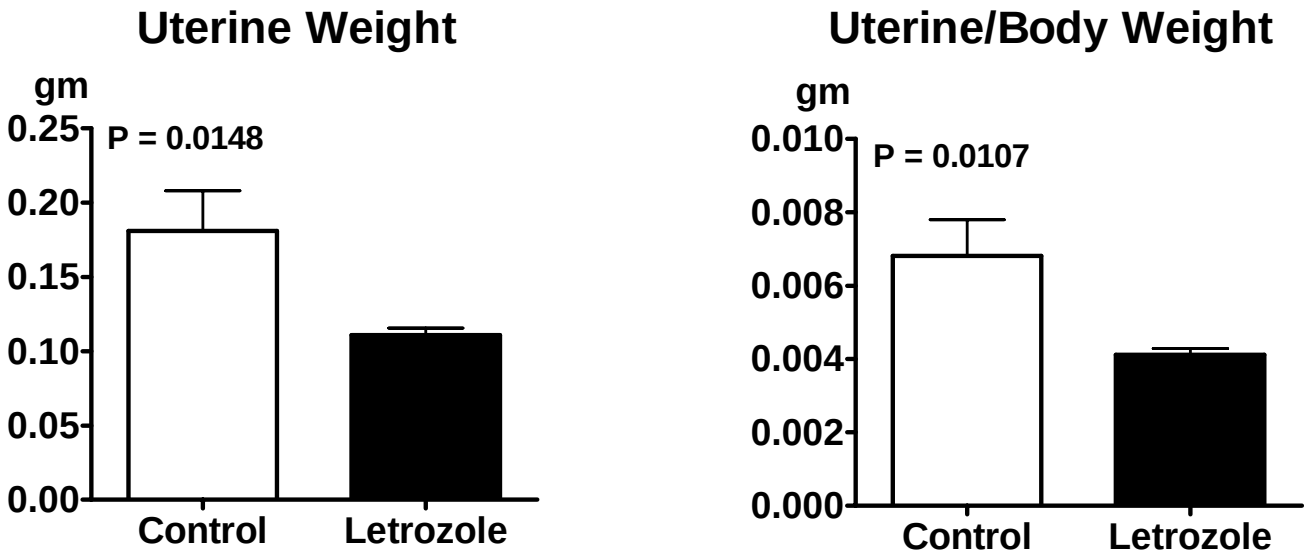



**Figure 28. Uterine weight and uterine weight/body weight in female nude mice after 21 weeks of treatment with letrozole or control. Uterine weight and uterine weight/body weight was significantly lower in the letrozole-treated mice compared to the control mice. P values calculated using Student's t-Test.**

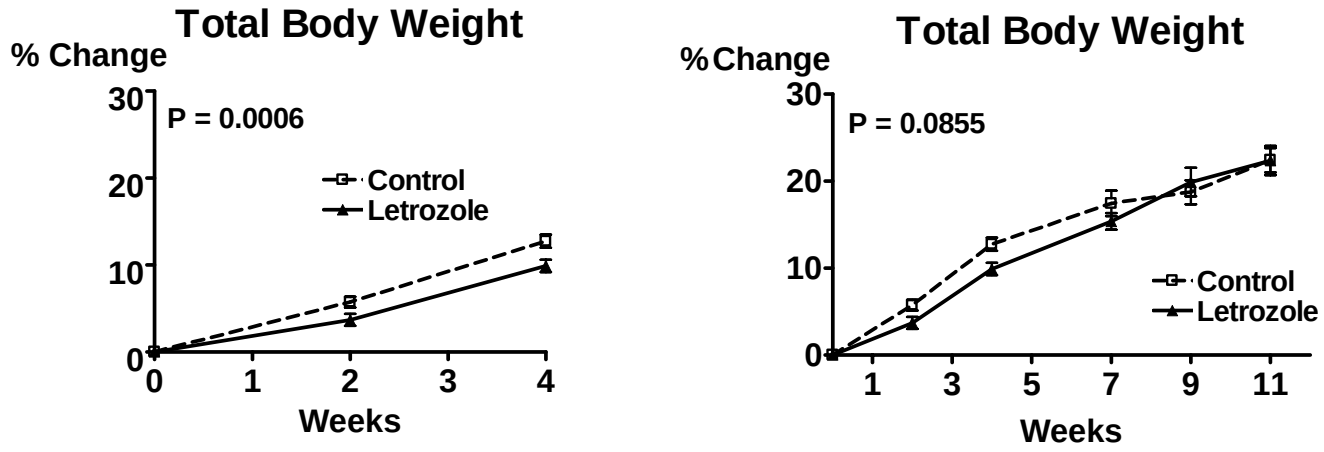



**Figure 29. Percent change of total body weight in female nude mice after 4 and 11 weeks of treatment with letrozole or control. The letrozole mice initially weighed significantly less than the control mice (p=0.0006) but caught up to the control mice by week 11 (p=0.0855). P values calculated using two-way ANOVA.**

## Key Research Accomplishments

- ***Publications:***

  - Meeting Report from Skeletal Complications of Malignancy IV; April 28-30, 2005 in Bethesda, Maryland, USA. This symposium was jointly sponsored by The Paget Foundation for Paget's Disease of Bone and Related Disorders, the National Cancer Institute and the University of Virginia School of Medicine. Authors: Robert L. Vessella, Theresa A. Guise, Edward S. Susman, Larry J. Suva, Gregory A. Clines, Scott L. Kominsky, Kristy L. Weber, John M. Chirgwin, Laurie K. McCauley and **Wende Kozlow**. Published on BoneKEy: www.bonekey-ibms.org.

  - **Kozlow W** and Guise TA. Breast Cancer Metastasis to Bone: Mechanisms of Osteolysis and Implications for Therapy. Journal of Mammary Gland Biology and Neoplasia. 2005; 10(2):169-80.

  - Guise TA, **Kozlow WM**, Heras-Herzig A, Padalecki SS, Yin JJ, Chirgwin JM. Molecular mechanisms of breast cancer metastases to bone. Clinical Breast Cancer. 2005; 5 suppl (2):S46-53.

  - **W.M. Kozlow**, K. Mohammad, R. McKenna, M. Niewolna and T.A. Guise. Aromatase inhibition results in lower bone density than ovariectomy in mice, an effect prevented by bisphosphonates. Journal of Bone & Mineral Research 2005; 20 (Suppl 2): S34.

  - **W. Kozlow**, K. Mohammad, R. McKenna, M. Niewolna, L. Suva, C. Rosen, T.A. Guise. Aromatase inhibition causes lower bone density than ovariectomy in mice, an effect prevented by bisphosphonates. Journal of Bone & Mineral Research 2005; 20 (Suppl 1): S313.

- ***Completed Courses:***

  - Spring 2005 School of Continuing & Professional Studies
    *BIMS 710 RESEARCH ETHICS S 1.0

  - Fall 2005 School of Continuing & Professional Studies
    *HES 700 INTRO TO BIOSTATISTICS A 3.0

- ***Certifications:***

  - Endocrine University: March 11-16, 2006 (certified by American College of Endocrinology (AACE))

- AACE Thyroid Ultrasound and FNA Biopsy Accreditation Course®: March 11-12, 2006 (certified by AACE)

- Certified Clinical Densitometrist (CCD): March 15, 2006 (certified by International Society for Clinical Densitometry)

- ***Additional Training:***

  - Mouse ovariectomy/sham surgery procedure
  - Mouse bone density and body composition analysis
  - Mouse x-ray analysis
  - Bone histomorphometry
    - ❖ trabecular bone volume analysis
    - ❖ osteoclasts, osteoblasts and adipocyte analysis
    - ❖ bone formation rate and mineral apposition rate analyses

- ***Awards:***

  - Women in Endocrinology 2006 Abstract Award in recognition of the abstract submitted to Endocrine Society 2006.

  - Award for Translational Cancer Research from the V Foundation – American Association for Cancer Research; awarded 04/19/05.

**Reportable Outcomes**

- ***Presentations:***

  - Blackard Symposium October 2005; Richmond, VA

- ***Abstracts (provided in the appendix):***

  - Skeletal Complications of Malignancy IV Meeting; April 2005; Bethesda, MD
  - University of Virginia Medicine Research Day; May 2005; Charlottesville, VA
  - American Society Bone & Mineral Research (ASBMR) annual meeting; September 2005; Nashville, TN
  - Endocrine Society annual meeting; June 2006; Boston, MA
  - ASBMR annual meeting; September 2006; Philadelphia, PA

- ***Employment:***

  - March 1, 2006: Director of the endocrinology elective for UVA medical students and medicine residents

- July 1, 2006: Assistant Professor of Research, Division of Endocrinology and Metabolism, Department of Medicine, University of Virginia (80% research and 20% clinical)

## Conclusion

Tamoxifen therapy is bone-sparing, but its use in breast cancer is rapidly superseded by AIs. Unlike tamoxifen, AI therapy for breast cancer results in high bone turnover. This leads to osteoporosis and fractures. It may increase breast cancer bone metastases. Women treated with AIs can expect to remain on therapy for a prolonged period of time. Therefore, it is important to assess the long-term consequences of AI therapy, including its effects on skeletal health. If AIs result in increased bone turnover and decreased BMD, concomitant treatment with antiresorptive agents must be considered. Our mouse model will 1) define the effect of a high bone turnover state induced by breast cancer therapy on the development and progression of breast cancer bone metastases and 2) test effective therapy to prevent increased bone turnover and breast cancer bone metastases. Preventing metastases to bone blocks the progression to Stage IV cancer, which is generally incurable. The findings from this multidisciplinary research protocol may reduce the morbidity from osteoporosis and bone metastases experienced by breast cancer patients.

## References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## Appendices

1. Abstract from the Skeletal Complications of Malignancy IV Meeting; April 2005; Bethesda, MD:

Aromatase Inhibition Results in Lower Bone Density Than Ovariectomy in Mice an Effect Prevented by Bisphosphonates

W.M. Kozlow, K. Mohammad, R. McKenna, M. Niewolna and T.A. Guise.

*Department of Internal Medicine, Division of Endocrinology and Metabolism, University of Virginia, USA*

Aromatase inhibitors have emerged as superior to tamoxifen to treat breast cancer. These drugs block estrogen synthesis by inhibiting the rate-limiting step in the conversion of testosterone and androstenedione to estradiol and estrone, respectively. This reduction in estrogen synthesis can be anticipated to increase bone resorption, thereby decreasing bone density. Clinical trials confirm that aromatase inhibitors reduce bone density in breast cancer patients. Bisphosphonates, inhibitors of bone resorption, may be useful to prevent bone loss due to aromatase inhibitor therapy. To study the effect of estrogen deficiency on bone mineral density (BMD) in mice, we performed 2 experiments. In the first study, 4-week-old female nude mice were randomized to bilateral ovariectomy or sham surgery (n = 12/group). BMD was measured at baseline and then every 2 weeks with GE Lunar PIXImus. At 8 weeks post-surgery, there was no difference in BMD in the ovariectomized mice compared to the sham control mice at any site: total body ($p = 0.6814$), spine ($p = 0.3398$), femur ($p = 0.3914$) and tibia ($p = 0.3093$). Next, we studied the effect of aromatase inhibitors +/- bisphosphonates on BMD. Forty 4-week-old female nude mice were randomized to 4 treatment groups: control (0.3% hydroxypropyl cellulose in PBS), letrozole (10 mcg SQ QD), zoledronic acid (5 mcg/kg SQ twice weekly) or letrozole (10 mcg SQ QD) plus zoledronic acid (5 mcg/kg SQ twice weekly). BMD was measured at baseline and then every 2 weeks for 13 weeks. Mice treated with letrozole alone had significantly lower BMD compared to mice treated with control at all sites: total body ($p < 0.0001$), spine ($p = 0.0002$), femur ($p = 0.0005$) and tibia ($p < 0.0001$). Mice treated with zoledronic acid had higher BMD compared to mice treated with control at all sites: total body ($p < 0.0001$), spine ($p < 0.0001$), femur ($p < 0.0001$) and tibia ($p < 0.0001$). Mice treated with letrozole plus zoledronic acid achieved the same bone density as mice treated with zoledronic acid alone at the spine ($p = 0.8546$) and tibia ($p = 0.2169$), but had greater bone density than mice treated with zoledronic acid alone at the femur ($p < 0.0001$) and total body ($p < 0.0023$). Thus, the aromatase inhibitor, letrozole, caused a reduction in bone density in female nude mice that was greater than that observed with ovariectomy alone. This bone loss was prevented by concomitant treatment with zoledronic acid. These results indicate that medical castration with aromatase inhibitors causes more profound bone loss than with ovariectomy. This may be due to the fact that aromatase inhibitors result in complete blockade of estrogen production compared to ovariectomy, where adrenal androgens may still be converted to estrogens by peripheral aromatase activity. Nonetheless, the significant bone loss induced by aromatase inhibition can be prevented with bisphosphonate therapy. Bisphosphonates may potentially be used for primary prevention against bone loss when therapy with an aromatase inhibitor is indicated.

2. Abstract from the University of Virginia Medicine Research Day; May 2005; Charlottesville, VA:

AROMATASE INHIBITION RESULTS IN LOWER BONE DENISTY THAN OVARIECTOMY IN MICE, AN EFFECT PREVENTED BY BISPHOSPHONATES

Wende Kozlow, Khalid Mohammad, Ryan McKenna, Maryla Niewolna and Theresa A. Guise.

Aromatase inhibitors (AIs) have emerged as superior to tamoxifen to treat breast cancer. These drugs block estrogen synthesis by inhibiting the rate-limiting step in the conversion of testosterone and androstenedione to estradiol and estrone, respectively. Reduction in estrogen synthesis can be anticipated to increase bone resorption, thereby decreasing bone mineral density (BMD). Bisphosphonates (BPs), inhibitors of bone resorption, may prevent bone loss due to AI therapy. To study the effect of estrogen deficiency on BMD in mice, we performed 2 experiments. In the first study, 4-week-old female nude mice were randomized to bilateral ovariectomy (ovx) or sham surgery (n=12/group). BMD was measured at baseline and then every 2 weeks with GE Lunar PIXImus. At 8 weeks post-surgery, there was no difference in BMD in the ovx mice compared to the sham mice at any site: total body (p=0.6814), spine (p=0.3398), femur (p=0.3914) and tibia (p=0.3093). Next, we studied the effect of AIs +/- BPs on BMD. Forty 4-week-old female nude mice were randomized to 4 treatment groups: control (0.3% hydroxypropyl cellulose in PBS), letrozole (10 mcg SQ QD), zoledronic acid (ZA) (5 mcg/kg SQ twice weekly) or letrozole (10 mcg SQ QD) plus ZA (5 mcg/kg SQ twice weekly). BMD was measured at baseline and then every 2 weeks for 13 weeks. Mice treated with letrozole alone had significantly lower BMD compared to mice treated with control at all sites: total body ($p<0.0001$), spine ($p=0.0002$), femur ($p=0.0005$) and tibia ($p<0.0001$). Mice treated with ZA had higher BMD compared to mice treated with control at all sites: total body ($p<0.0001$), spine ($p<0.0001$), femur ($p<0.0001$) and tibia ($p<0.0001$). Mice treated with letrozole plus ZA achieved the same bone density as mice treated with ZA alone at the spine and tibia, but had greater bone density than mice treated with ZA alone at the femur ($p<0.0001$) and total body ($p<0.0023$). The AI caused a reduction in BMD in female nude mice that was greater than that observed with ovx alone. This bone loss was prevented by concomitant treatment with ZA. These results indicate that medical castration with AIs causes more profound bone loss than with ovx. BPs may potentially be used for primary prevention against bone loss when therapy with an AI is indicated.

3. Abstract from the American Society Bone & Mineral Research (ASBMR) annual meeting; September 2005; Nashville, TN:

Aromatase Inhibition Causes Lower Bone Density Than Ovariectomy in Mice, an Effect Prevented by Bisphosphonates.

W. Kozlow[1], K. Mohammad[1], R. McKenna[1], M. Niewolna[1], L. Suva[2], C. Rosen[3], T.A. Guise[1].

[1]Internal Medicine, University of Virginia, Charlottesville, VA, USA, [2]Orthopaedic Surgery, University of Arkansas, Little Rock, AR, USA, [3]Jackson Laboratory, Bar Harbor, ME, USA.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block estrogen synthesis by inhibiting the conversion of testosterone and androstenedione to estradiol and estrone. Increased bone resorption and decreased bone mineral density (BMD) are predicted consequences. We hypothesized that bisphosphonates (BPs) may prevent bone loss from AI therapy. We studied the effect of estrogen deficiency on bone remodeling in 4-week-old female nude mice that underwent ovariectomy (ovx) or sham surgery. Ovx and sham mice did not differ in BMD (assessed by DXA) or in histomorphometric assessment of trabecular bone volume. Next, to study the effect of AIs +/- BPs on bone remodeling, 4-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole (10 mcg/d) + ZA (5 mcg/kg twice weekly) or control. Mice treated with letrozole alone had lower BMD compared to control mice ($p<0.0001$; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control mice ($p<0.0001$; total body, spine, femur and tibia). Mice treated with letrozole plus ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the femur ($p<0.0001$) and total body ($p<0.0023$). MicroCT analysis of the proximal tibia showed no difference in bone volume (BV/TV), structural model index, or trabecular number, thickness or spacing in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV and trabecular number and thickness, and the structural model index indicated that the bone structure was unusually solid. Dynamic bone histomorphometry of the lumbar spine demonstrated decreased bone formation and mineral apposition rates in mice treated with letrozole, ZA or the combination compared to control. Serum testosterone concentrations were increased in mice treated with letrozole compared to control. Serum IGF-1 concentrations were similar in all groups. These data indicate that aromatase inhibition with letrozole caused lower BMD in female nude mice than that observed with ovx. The greater effect of AIs compared to ovx may be due to reduced adrenal androgen conversion to estrogen. ZA prevented AI-induced bone loss, but microCT and dynamic bone histomorphometry suggest reduced bone remodeling. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

4. <u>Abstract for the Endocrine Society annual meeting; June 2006; Boston, MA:</u>

Aromatase Inhibition Results in Loss of Bone Mineral Density, an Effect Prevented by Bisphosphonates

Wende M Kozlow[1], Khalid Mohammad[1], Ryan McKenna[1], Maryla Niewolna[1], Larry J Suva[2] and Theresa A Guise[1]. [1]Department of Endocrinology and Metabolism, University of Virginia, Charlottesville, Virginia, United States, 22908 and [2]Department of Orthopaedic Surgery, University of Arkansas for Medical Sciences, Little Rock, Arkansas, United States, 72205.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block the conversion of androstenedione and testosterone into estrone and estradiol. Anti-cancer therapies that suppress estrogen lead to increased bone resorption and the loss of bone mineral density (BMD). Cancer treatment-induced bone loss will likely become one of the most common skeletal complications of malignancy. We hypothesized that bisphosphonate (BP) treatment may prevent increased bone resorption from AI therapy, and impact bone formation.

To study the effect of AIs +/- BPs on bone remodeling, 4-week-old female nude mice were treated with letrozole (10 mcg/d), zoledronic acid (ZA) (5 mcg/kg twice weekly), letrozole + ZA, or control. BMD was assessed by DXA. Mice treated with letrozole alone had lower BMD compared to control ($p<0.0001$; total body, spine, femur and tibia). Mice treated with ZA alone had higher BMD compared to control ($p<0.0001$; total body, spine, femur and tibia). Mice treated with letrozole + ZA achieved the same BMD as mice treated with ZA alone at the spine and tibia, but had greater BMD than mice treated with ZA alone at the femur ($p<0.0001$) and total body ($p<0.0023$). MicroCT analysis of the proximal tibia showed no difference in bone volume (BV/TV), structural model index (SMI), or trabecular number (Tb.N), thickness (Tb.Th) or separation in mice treated with letrozole alone compared to control. Treatment with ZA (+/- letrozole) resulted in a significant increase in BV/TV, Tb.N and Tb.Th, and the SMI indicated that the bone structure was unusually solid. Dynamic bone histomorphometry of the lumbar spine demonstrated decreased bone formation and mineral apposition rates in mice treated with letrozole, ZA or the combination compared to control.

To assess the effect of letrozole on bone formation, calvaria obtained from 4-day-old mice were cultured for 7 days with media (BGJ) alone, positive control (insulin) or letrozole. Histomorphometry demonstrated that letrozole did not stimulate new bone formation and, when combined with insulin, did not inhibit new bone formation.

Letrozole decreased BMD in female nude mice, an effect prevented by concomitant treatment with ZA. MicroCT and histomorphometry indicate that the mechanism involves reduced bone remodeling with no direct effect of the treatment on bone formation. BPs may be useful to prevent AI-induced bone loss, but further studies are needed to assess the effects of these treatments on bone quality.

5. Abstract for the ASBMR annual meeting; September 2006; Philadelphia, PA:

Aromatase Inhibition Results in Gain of Bone Mineral Density in the Spine and Femur in Female Nude Mice

W. Kozlow[1], K. Mohammad[1], C. R. McKenna*[1], H. Walton*[1], M. Niewolna*[1], J. D. Dilley*[2], L. J. Suva[2], T. A. Guise[1]. [1]Internal Medicine, University of Virginia, Charlottesville, VA, USA, [2]Orthopaedic Surgery, University of Arkansas for Medical Sciences, Little Rock, AR, USA.

Aromatase inhibitors (AIs), effective treatment for breast cancer, block the conversion of androstenedione and testosterone into estrone and estradiol. Suppression of estrogen leads to increased bone resorption and the loss of bone mineral density (BMD). Therefore, cancer treatment-induced bone loss will likely become one of the most common skeletal complications of malignancy. We hypothesized that the AI letrozole would result in loss of BMD in female nude mice.

Four-week-old female nude mice were treated with letrozole (10 mcg/d) or control. BMD was assessed at baseline and every 2 weeks thereafter. Surprisingly, mice treated with letrozole had increased BMD compared to control at the mid femur ($p=0.0030$) and spine ($p=0.0002$). There was no difference in BMD between control and letrozole-treated mice at the total body, proximal femur or proximal tibia. MicroCT analysis of the femur after 4 weeks of treatment did not show a significant difference in trabecular bone volume (BV/TV), although a trend toward increased BV/TV in the letrozole-treated mice was observed ($p=0.0659$). However, 4 weeks of treatment with letrozole induced marked increases in skeletal microarchitecture. Significant increases in connectivity density ($p=0.0012$) and trabecular number ($p=0.0538$), thickness ($p=0.0280$) and separation ($p=0.0348$) were observed in the femurs of letrozole-treated mice, but not in the tibias. Interestingly, these data differ from published data using immunocompetent aromatase null mice, suggesting that differences in T-cell populations in nude mice may account for these distinct effects on bone density and architecture.

In a separate experiment, 4-week-old female nude mice were treated with the bisphosphonate zoledronic acid (ZA) (5 mcg/kg) twice weekly +/- letrozole (10 mcg/d) for 14 weeks. Mice treated with letrozole + ZA had increased BMD at the proximal femur ($p<0.0001$) and total body ($p=0.0003$) compared to ZA alone but, by histomorphometric analysis, bone formation rates were not increased. Similarly, letrozole did not stimulate or inhibit osteoblast number or bone formation in ex-vivo cultures of neonatal mouse calvariae.

In conclusion, letrozole increased BMD at the spine and mid femur and increased trabecular architecture in the femur. This effect, pronounced in the presence of bisphosphonate treatment, was not due to a direct effect of letrozole on bone formation. Unlike in intact immunocompetent mice, letrozole appears to have site-specific effects on the skeletons of nude mice.